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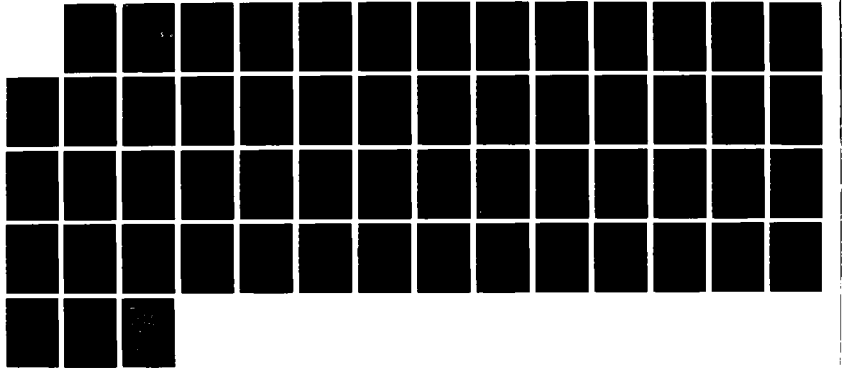

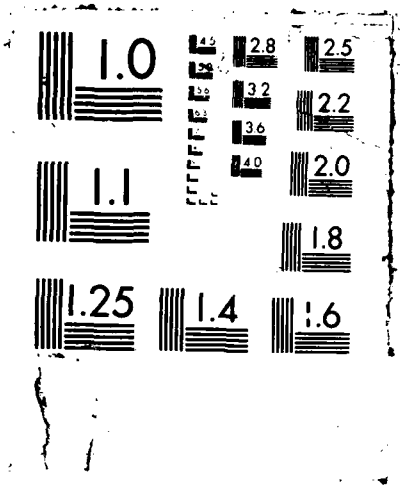
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THE DESIGN, SYNTHESIS AND SCREENING OF POTENTIAL PYRIDINIUM OXIME PRODRUGS

ANNUAL REPORT

RONALD T. BORCHARDT

JOHN E. SIMMONS

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

In an attempt to improve the CNS delivery of quaternary pyridinium oxime regenerators of acetylcholinesterase (AChE), we have initiated chemical and biochemical studies on structural analogs and prodrug forms of N-methylpyridinium 2-carbaldoxime (2-PAM, 7). Over the past year we have concentrated our efforts on synthesizing two basic types of Pro-PAM derivatives. Series I are dihydropyridinium oximes 1 and 2 which possess electron withdrawing substituents in the 3- or 5-position. These compounds

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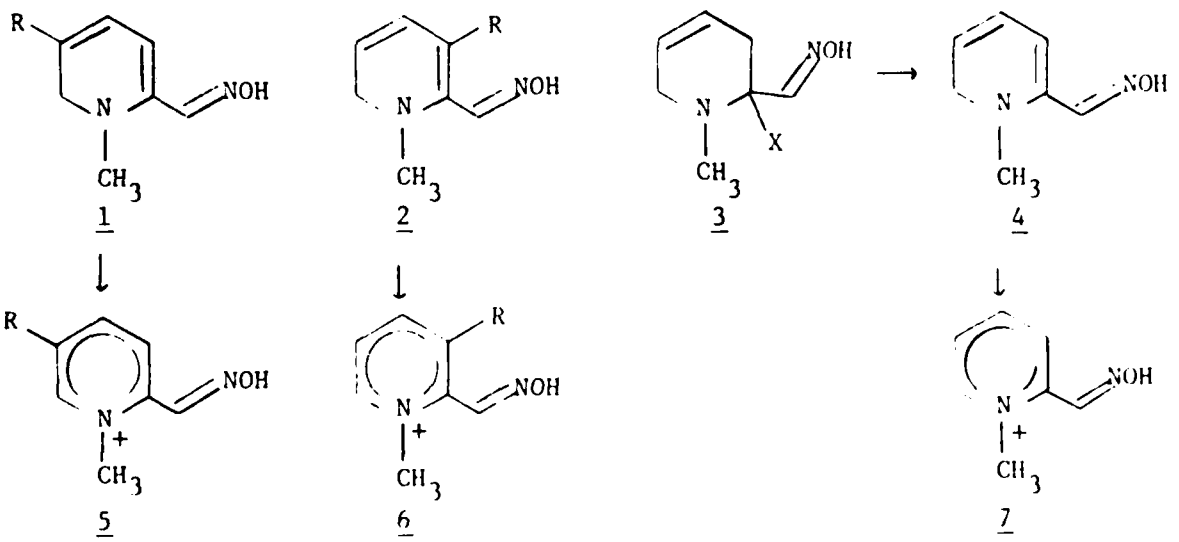
require oxidation (latentation) to generate the substituted 2-PAMs 5 and 6. It is hoped that the electron withdrawing substituent will stabilize the dihydropyridine structure and produce a slow in vitro conversion. Series II are tetrahydropyridinium oximes 3 which possess a labile ring substituent. These masked prodrugs (double latentation) are addition products of dihydropyridinium oxime 4 (Pro-2-PAM) and require a two step (elimination and oxidation) conversion to the active quaternary oxime 7 (2-PAM).



Series I Series II

In series I the 5-substituted-2-PAMs 5 (R=CN,I) and a 3-substituted-2-PAM 6 (R=I) have been synthesized and characterized. In series II the tetrahydropyridinium oximes 3 (X=CN,SCN) have been made and characterized.

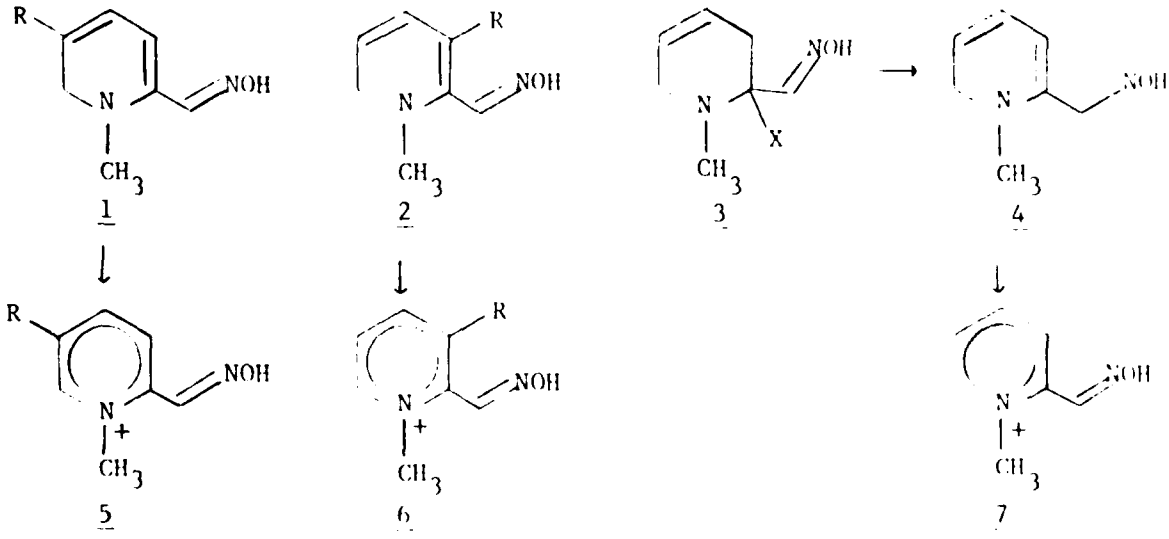
A screening assay employing immobilized AChE has been developed. AChE covalently attached to functionalized polyethylene beads shows good esterase activity and is stable when stored at -16°C for up to two months. The AChE activity of the immobilized enzyme can be continuously monitored spectrophotometrically in a closed loop fashion using acetylthiocholine and dithiobis (nitrobenzoic acid). The assay allows for independent inactivation and reactivation of AChE, followed by the determination of regenerated AChE activity. Both charged (2-PAM, TMB-4) and uncharged (MINA) regenerators were used to evaluate and standardize the assay. N-Methyl-3-iodo-2-pyridinium carbaldoxime (6, R=I) was found to be approximately 50% as active as 2-PAM in the immobilized AChE screening assay.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

Summary

In an attempt to improve the CNS delivery of quaternary pyridinium oxime regenerators of acetylcholinesterase (AChE), we have initiated chemical and biochemical studies on structural analogs and prodrug forms of N-methylpyridinium 2-carbaldoxime (2-PAM, 7). Over the past year we have concentrated our efforts on synthesizing two basic types of Pro-PAM derivatives. Series I are dihydropyridinium oximes 1 and 2 which possess electron withdrawing substituents in the 3- or 5-position. These compounds require oxidation (latentation) to generate the substituted 2-PAMs 5 and 6. It is hoped that the electron withdrawing substituent will stabilize the dihydropyridine structure and produce a slow *in vivo* conversion. Series II are tetrahydropyridinium oximes 3 which possess a labile ring substituent. These masked prodrugs (double latentation) are addition products of dihydropyridinium oxime 4 (Pro-2-PAM) and require a two step (elimination and oxidation) conversion to the active quaternary oxime 7 (2-PAM).



Series I Series II

A-1

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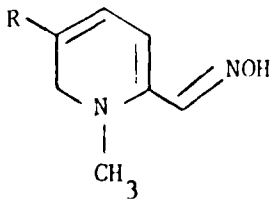
Appendix

A. Abstract, Presentation at the 67th Annual Meeting, FASEB, Chicago, IL, April 10-15 (1983) Abstr. #2135 [Federation Proc.42, 657 (1983)].

A. Problem

1. Design and Synthesis of Pro-PAM Agents

The overall objective of the project is to improve central nervous system (CNS) delivery of quaternary pyridinium oxime regenerators of acetylcholinesterase (AChE). The specific chemical problem is to design and synthesize nonquaternary, lipophilic prodrugs of pyridinium oximes which can be easily transformed *in vivo* into active quaternary regenerators. There are two basic designs which are currently being investigated:
a) Dihydropyridine carboxaldoximes 1 and 2 substituted at the 3- or 5-position with an electron withdrawing group (R) - activation of these compounds to quaternary pyridinium oximes requires simple oxidation.
b) Tetrahydropyridine oximes 3 substituted at the 2-position with a labile leaving group (X). Activation of these analogs requires elimination (-HX) followed by oxidation to generate quaternary pyridinium oximes.



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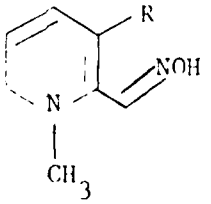

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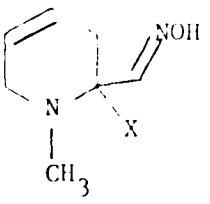

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The rate and efficiency by which the parent quaternary pyridinium oxime of each Pro-PAM analog is generated will need to be determined in order to evaluate their suitability for therapeutic use. The pKa's and partition coefficients of the parent pyridinium oximes and prodrug forms will need to be measured in order to evaluate the prodrug's ability to penetrate membranes and it's ability to react as a nucleophile with the phosphorylated enzyme.

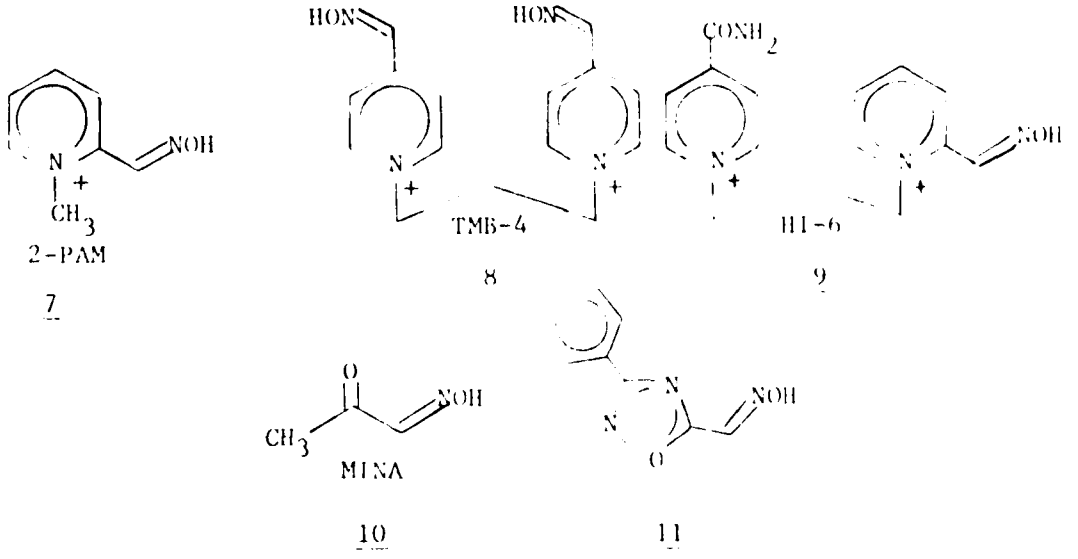
2. Biological Testing

All Pro-PAM derivatives and their parent pyridinium oximes require evaluation as regenerators of AChE deactivated with an organophosphate. It is necessary to devise an *in vitro* screening assay, which can be used aerobically to evaluate the efficacy of the parent quaternary pyridinium oximes and anaerobically to evaluate their prodrug forms. To test the effectiveness of the proposed oximes on the survival of organophosphate treated animals it will be necessary to set up an *in vivo* screening protocol.

B. Background

Organophosphates as a class owe their toxicity to their ability to react covalently with the esteratic site of AChE. AChE is the enzyme responsible for hydrolyzing the neurotransmitter acetylcholine once it has functioned in a neurochemical event. The phosphorylated enzyme can be reactivated by a variety of agents. There is, however, a competing "aging" process whereby the inactivating phosphoryl group either migrates to an adjacent amino acid residue[1] or is partially hydrolyzed.[2,3] Phosphorylated AChE which has undergone this "aging" process is not easily reactivated. Thus rapid reactivation of the poisoned enzyme in all effected tissues is highly desirable.

I. B. Wilson discovered 2-PAM (7), which is now one of the most widely used and therapeutically effective AChE regenerators.[4] Wilson postulated that the electrostatic attraction of the quaternary nitrogen helped orient the oxime moiety toward the phosphorylated esteratic site (Figure 1).[4-7]



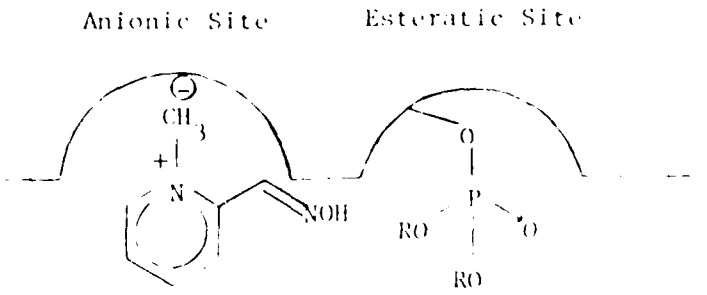


Figure 1

Since the discovery of 2-PAM there have been a variety of other organic oximes which have been synthesized and screened for AChE regenerating activity. The charged bis oximes TMB-4 (8) and HI-6 (9) have both proven to be potent reactivators, but suffer from poor tissue penetration, short serum half lives and toxicity problems.[8-11] Neutral oximes such as MINA (10) and 5-hydroxyiminomethyl-3-phenyl-1,2,4-oxadiazole (11) have shown much less potent regenerator ability, but do possess better lipid solubility.[12-14]

Investigations into the structure activity relationships of substituted 2-PAMs has produced some interesting information. In general, electron withdrawing substituents shift the pKa of the pyridinium oxime to values below the optimum range of 7.4-7.8 and electron donating substituents shift it higher.[4,15] Some 5-substituted 2-PAMs (Cl, CH_3) were approximately as effective as 2-PAM in whole animal survival studies even though in vitro testing showed them to be less effective at regenerating deactivated AChE.[16]

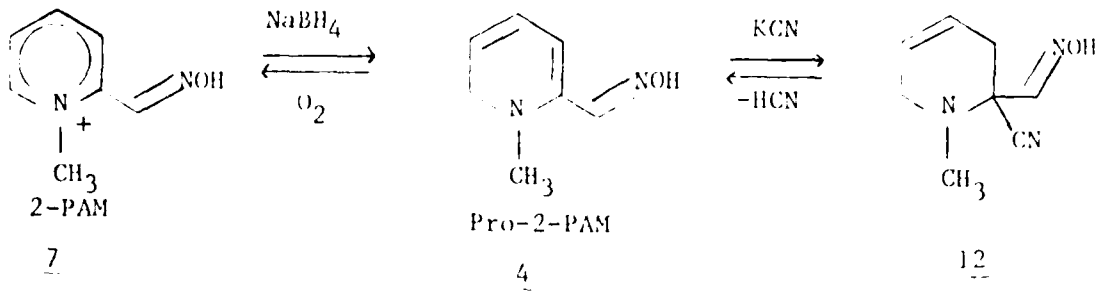
These results indicated that the active site can tolerate minor structural changes in 2-PAM and further that desirable solubility characteristics might be incorporated into the molecule without significantly reducing its reactivating capabilities. In an attempt to increase the lipid solubility of 2-PAM, N-dodecyl-2-pyridinium carbaldoxime (2-PAD) was made. It possessed increased lipid solubility, but proved to be far less effective than 2-PAM at in vitro reactivation.[17]

Only trace amounts of 2-PAM can be detected in the CNS following i.v. injection.[1] Due to its high water solubility the intact drug is rapidly eliminated from the body with an observed half life in humans of less than one hour. Blood levels fall below the therapeutically effective range after 1-2 hours.[18-20] Significant brain levels of 2-PAM can only be achieved by intraventricular injections of the drug.[21,22]

Asphyxiation due to suppression of the central respiratory center is the ultimate cause of death in mammals exposed to anticholinesterase agents.[23,24] The lipid permeability of many organophosphates allows them to penetrate many body tissues including the CNS which are impermeable to a charged molecule like 2-PAM. Therefore the need to regenerate AChE in the CNS is obvious.

The delivery of 2-PAM to the highly lipid CNS was a problem which resisted solution until the pioneering work of Shek, Bodor and Higuchi.[25-28] The University of Kansas group synthesized a prodrug of 2-PAM. Working on the hypothesis that a tertiary amine would have little difficulty penetrating the CNS, they synthesized a partially reduced form of 2-PAM which was a latent quaternary amine. Figure 2 illustrates how they trapped the reduced

form of 2-PAM as a cyanide addition product 12 which upon careful decomposition afforded Pro-2-PAM (4). The pKa of 4 was determined to be 6.3 which was good for favorable physiological partitioning. Much like the NAD-NADH redox system, they found that in vivo oxidation of 4 to 2-PAM (7) took place in approximately 1 minute.[27] This was sufficient time to allow the drug to cross the blood-brain barrier producing a 13 fold increase in brain levels of 2-PAM.[28]



2-PAM
7

Pro-2-PAM
4

12

Figure 2

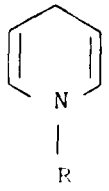
C. Approach

1. Prodrug Design

Our current work at the Universty of Kansas intends to build on and exploit the initial findings of Shek, Bodor and Higuchi.[25-28] We have over the past year focused our efforts on two approaches.

a. Series I - Dihydropyridinium oximes

In the first series of compounds the intent was to reduce measurably the rate of oxidation of a dihydropyridinium oxime to its active parent quaternary form by stabilizing the dihydro-structure with electron withdrawing substituents. It is known from the literature that an electron withdrawing group in the 3- or 5- position stabilized the dihydropyridines 14 and 15, relative to 13,through electron delocalization.[29]



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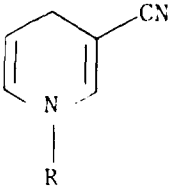

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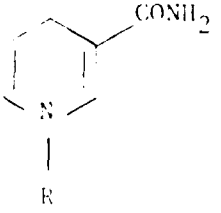

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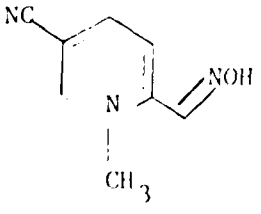
By synthesizing 3- and 5-substituted Pro-2-PAMs 16-19, it is hoped that the



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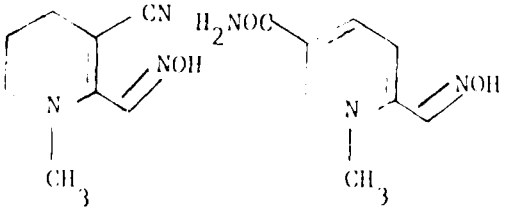

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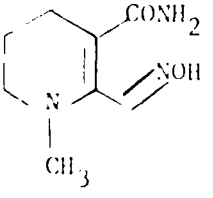

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rate of conversion from prodrug to the active quaternary pyridinium oxime forms can be slowed thus allowing more time for partitioning into the CNS compartment (Figure 3). There may very well be a trade off between reduced reactivating ability of the substituted pyridinium oxime and increased tissue permeability of the prodrug form. However, this can only be determined by in vivo testing.

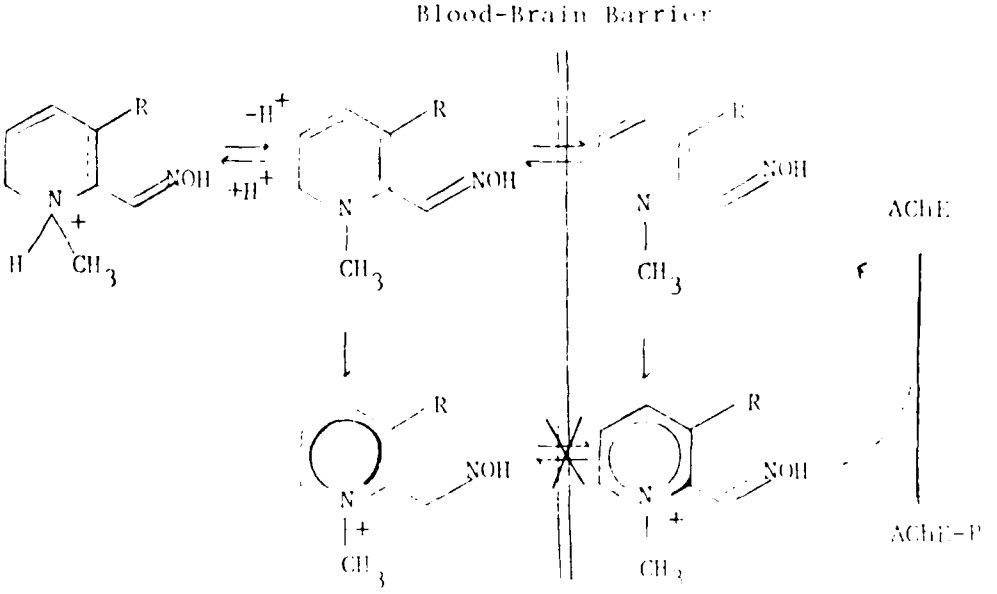
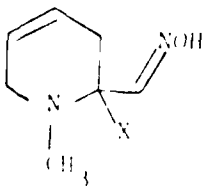


Figure 3

b. Series II - Tetrahydropyridinium Oximes

The second series of compounds we have examined this past year are prodrugs which require a two step conversion to the active quaternary pyridinium oxime - double latentation. Shek et al.[26] in their synthesis of Pro-2-PAM trapped the dihydropyridinium structure as a cyanide addition product 12 (Figure 2). Our intent is to take advantage of this trapping mechanism using nucleophiles (X) such as SCN, SO_3H, I, Br and OCN, which we hope will be as labile, but less toxic than cyanide. These doubly latent structures 3 could generate Pro-2-PAM (4) in vivo.



3 X = CN, SCN, SO_3H, I, Br, OCN

Figure 4 illustrates how the chances of a neutral species crossing membranes otherwise impermeable to the charged pyridinium oximes is effectively increased. Our initial thrust has been to synthesize doubly latent forms of 2-PAM since it's regenerating capabilities and pharmacokinetics have been extensively documented in the literature.

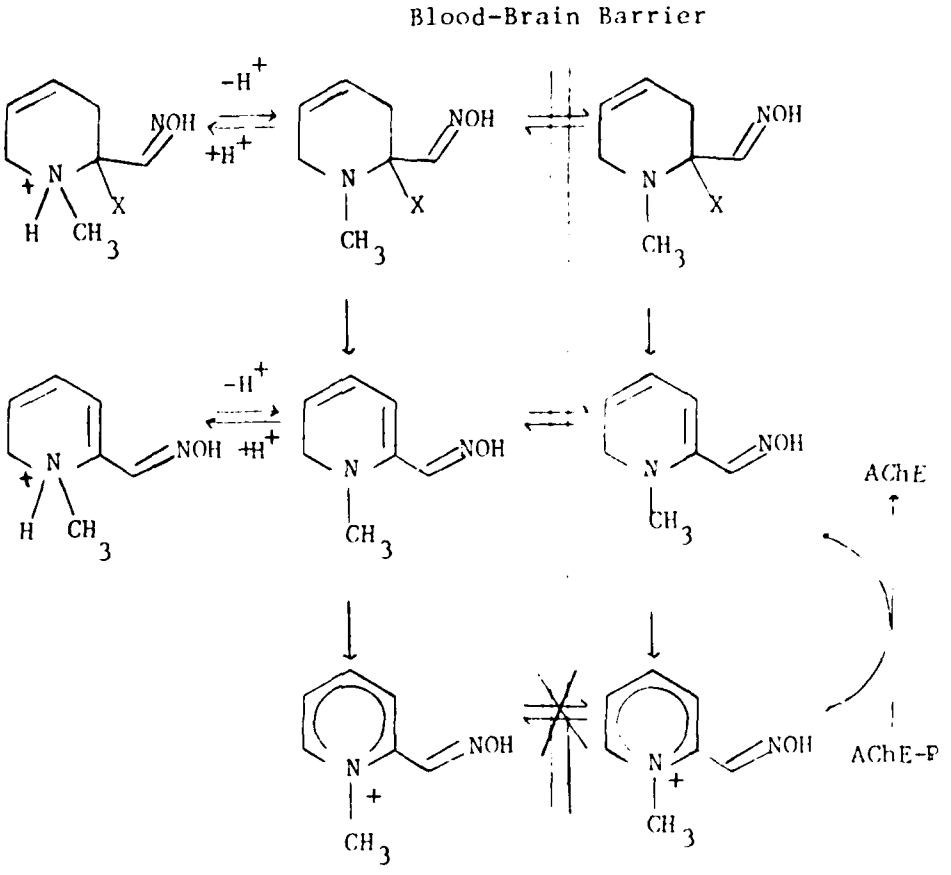


Figure 4

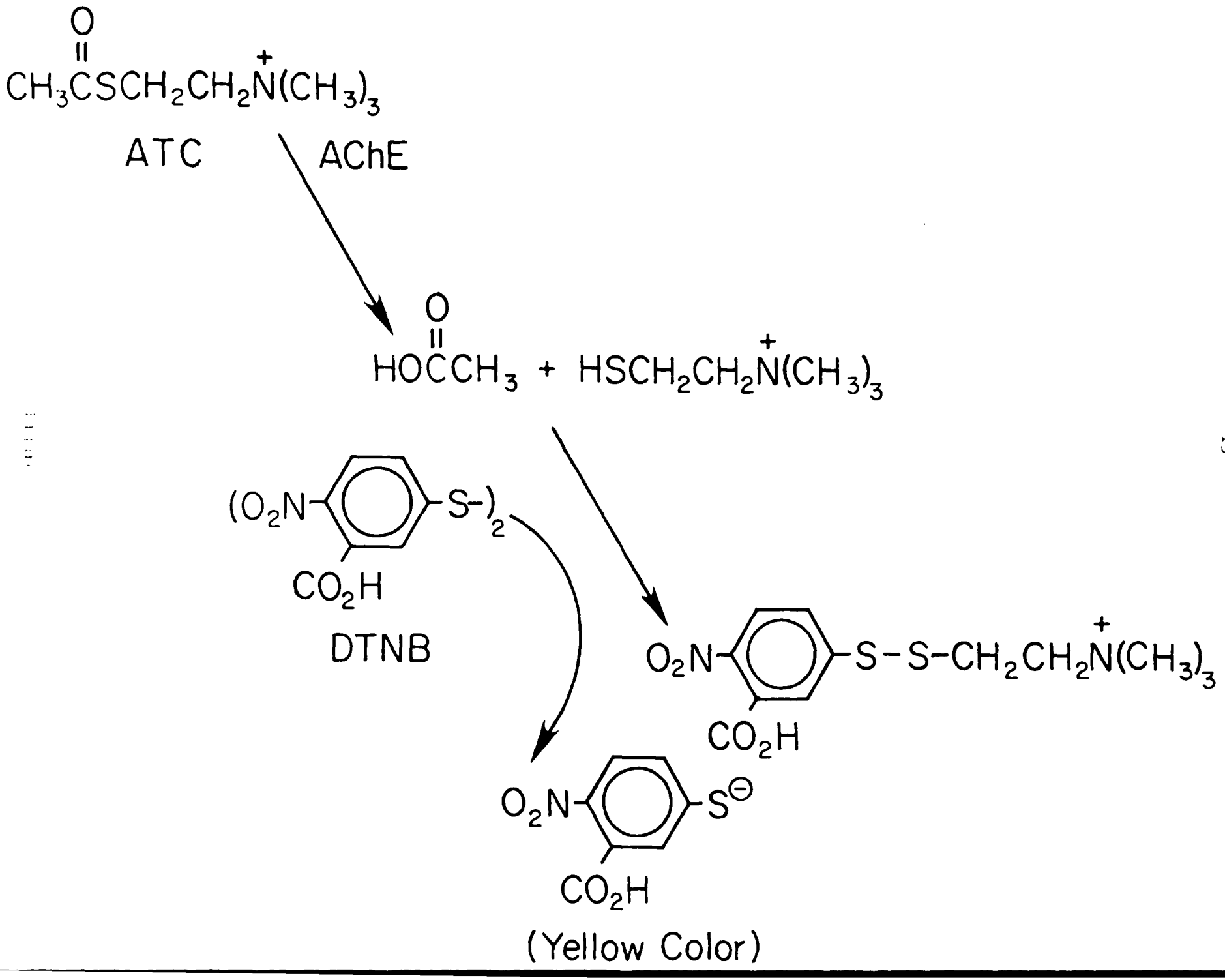
2. Biological Evaluations

a. In Vitro Assay

We have focused our attention in the past year on developing a reliable, workable in vitro assay as a preliminary screening technique for the AChE regenerators being synthesized in our laboratories. The basic Ellman technique (Figure 5) appeared to be a viable assay which did not require any dedicated equipment other than a spectrophotometer.[30] The intent was to have a rapid and efficient assay to be used as a primary screen for the parent quaternary pyridinium oximes. In this manner the quaternary oximes which exhibited poor or negligable reactivation capabilities could be identified which would allow us to concentrate our efforts on synthesizing prodrugs forms of the most active regenerators. In addition many of the prodrugs are oxygen sensitive which requires that the assay be adaptable to anaerobic conditions. All of the above requirements (i.e. rapid, reliable, sensitive and adaptable to anaerobic conditions) led us to the Ellman technique to measure AChE activity (Figure 5) and the use of an immobilized AChE.

Figure 5. The Ellman Technique for Measuring AChE Activity

ATC - Acetylthiocholine; DTNB - 5,5'-Dithiobis(2-Nitrobenzoic Acid)

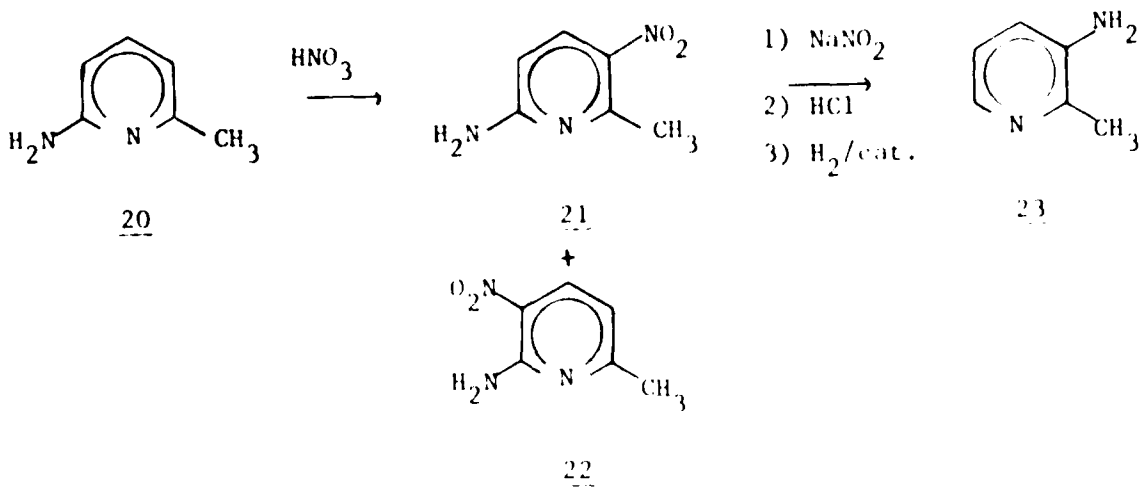

ATC
AChE
DTNB
(Yellow Color)

D. Results

1. Syntheses

a. Series I - Dihydropyridinium Oximes

Our original strategy for the synthesis of the 3-cyano-substituted pyridinium oxime was to prepare the intermediate 3-amino-2-picoline (23) (Figure 6).[31] However, nitration of 6-amino-2-picoline (20) produced not only the desired 3-nitro-compound 21, but also 6-amino-5-nitro-2-picoline (22). The overall yield was reasonable (80%), however, purification and separation of the isomers proved to be difficult, which caused us to abandon this approach.



20 21 + 22 23

Figure 6

Figure 7 outlines the alternate approach which was undertaken for the synthesis of the 3- and 5- cyano-substituted pyridinium oximes 33 and 34. By modifying a literature preparation for 5-iodo-2-picoline(25), we obtained not only the desired 5-iodo-2-picoline (25) but also the 3-iodo-compound 26. The overall yield was 30% with the isomers in roughly equal proportions. The iodopicolines could be separated chromatographically and each isomer was characterized spectrally. Due to the volatility of these compounds and difficulty in separation it was more convenient to work with the mixture. Using the method of Craig[33], a CuCN displacement on 25 and 26 yielded the 5- and 3-cyanopicolines 27 and 28 (75% combined yield). Here again the compounds could only be separated by chromatographic techniques. 5-Cyano-2-picoline (27) was obtained as pure material, but the 3-cyano-isomer 28 was always contaminated with traces of the 5-isomer. Despite the lack of literature precedence we attempted an I_2/DMSO oxidation[15] of the cyano-picolines. 5-Cyano-2-picoline (27) was oxidized to aldehyde 29, however, 3-cyano-2-picoline (28) did not yield the corresponding aldehyde 30. The yield (55%) in the oxidation step was only modest, however, it appeared to be the most direct route to the desired intermediate 29. The 5-cyano-2-picolinealdehyde (29) was then condensed with hydroxylamine under neutral conditions to give oxime 31 in good yields (70%). Quaternization of 5-cyano-2-pyridine carbaldoxime 31 to form 33 proved to be more difficult than anticipated. The cyano moiety delocalized electrons so effectively that the methylation required a 20-50 fold excess of methyl iodide in a sealed tube at 80-100°C. In addition the reaction was accompanied by formation of elemental iodine (methyl iodide decomposition) unless run under nitrogen. Dimethylsulfate would alkylate the ring nitrogen under somewhat less forcing conditions, but conversion to a halide counter ion and purification were more difficult.

Both the 3- and 5-iodo-2-picolines (25 and 26) were oxidized by I_2/DMSO to afford the corresponding aldehydes 37 and 38 (54% total yield). It was interesting that despite having a larger atom at the 3-position , 3-iodo-2-picoline (26) could be oxidized, whereas the 3-cyano-compound 28 could not. The implication was that electronic rather than steric factors were involved. The iodopicolinealdehydes 37 and 38 were easier to separate chromatographically than the iodopicolines 25 and 26. The iodopicoline-aldehydes 37 and 38 were successfully condensed with hydroxylamine and methylated with methyliodide to afford 3- and 5-iodopyridinium oximes 41 and 42.

The synthesis of the 3-cyano-substituted oxime 34 has not yet been completed, but should be achievable from the 3-iodopicolinealdehyde 38 which upon treatment with CuCN yields the 3-cyano-aldehyde 30. The yield is poor (10%), but optimization of this step is being attempted.

In an attempt to find more direct synthetic routes to the 3- or 5-cyano substituted pyridinium oximes we investigated carbanion additions to 3-cyano-pyridine (45). Based on literature precedence[34] we treated 3-cyanopyridine (45) with methyllithium. When the addition rate, reaction temperature and time were carefully controlled, significant amounts of 5-cyano-2-picoline (27) could be obtained. Under none of the reaction conditions employed could we isolate any 3-cyano-2-picoline (26).

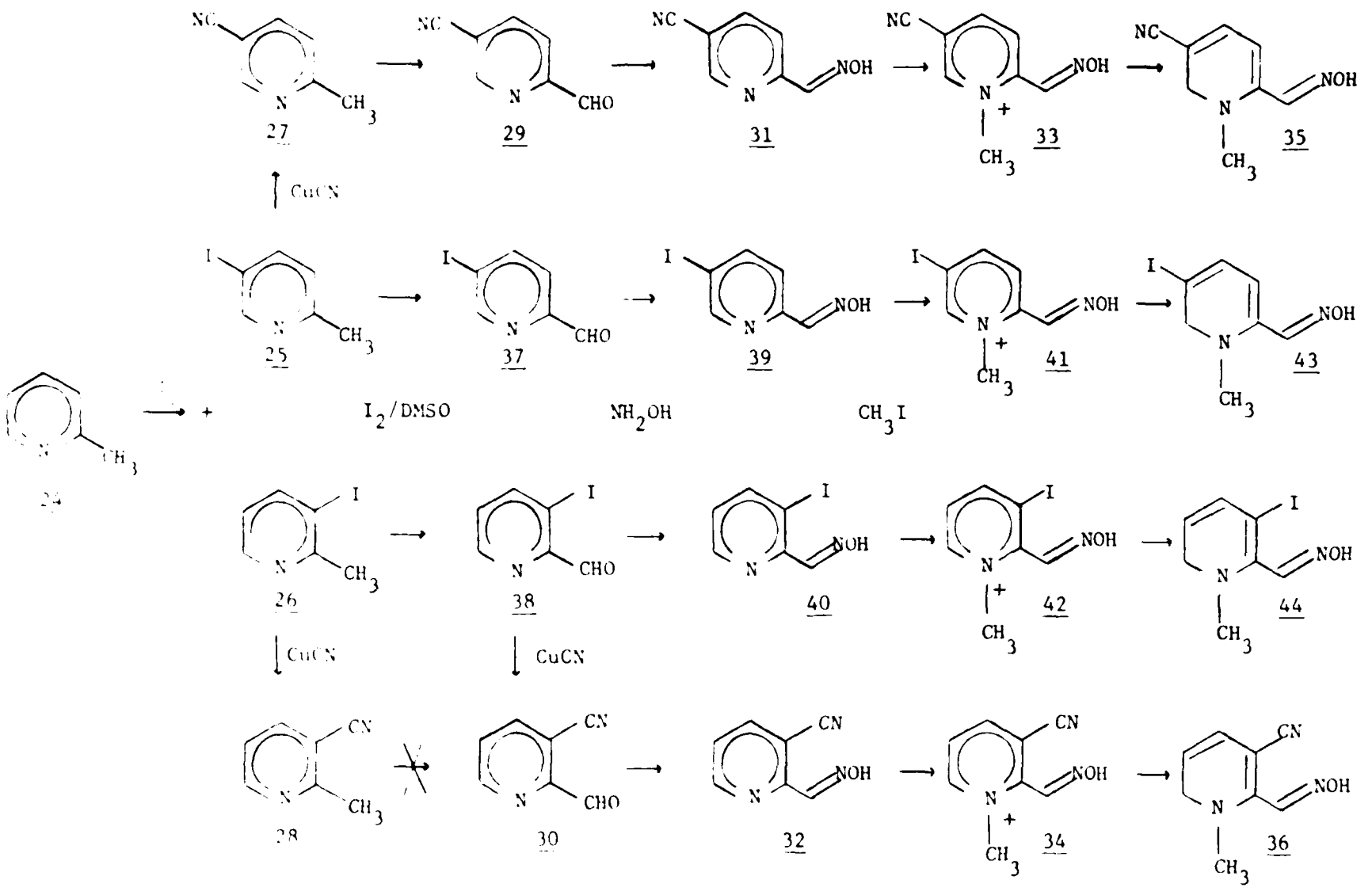

NC
CH3
27
CHO
29
NOH
31
33
35
CuCN
I
25
37
39
41
43
I2/DMSO
NH2OH
CH3I
24
26
38
40
42
44
CN
28
30
32
34
36

Figure 7

An extension of the technique to include the addition of a "formyl equivalent" (2-Lithio-1,3-dithiane 46) gave a mixture of 2,3- and 2,5-addition products 47 and 48 (Figure 8). At -40°C the 2,5-isomer 47 was formed almost exclusively. As the temperature of the addition was increased to -10°C more of the 2,3-isomer 48 was produced, but never exclusively. Further the 2,5-isomer 47 could be hydrolyzed to 5-cyano-2-picolinealdehyde (29) in good yields (40-60%) using mercuric chloride. The 2,3 isomer 48, however, could not be successfully deprotected to yield the desired aldehyde 30 using $HgCl_2/HgO$ in methanol/water,[35] acetonitrile/water, tetrahydrofuran/water[36] or $CuCl_2/CuO$ in acetone.[37]

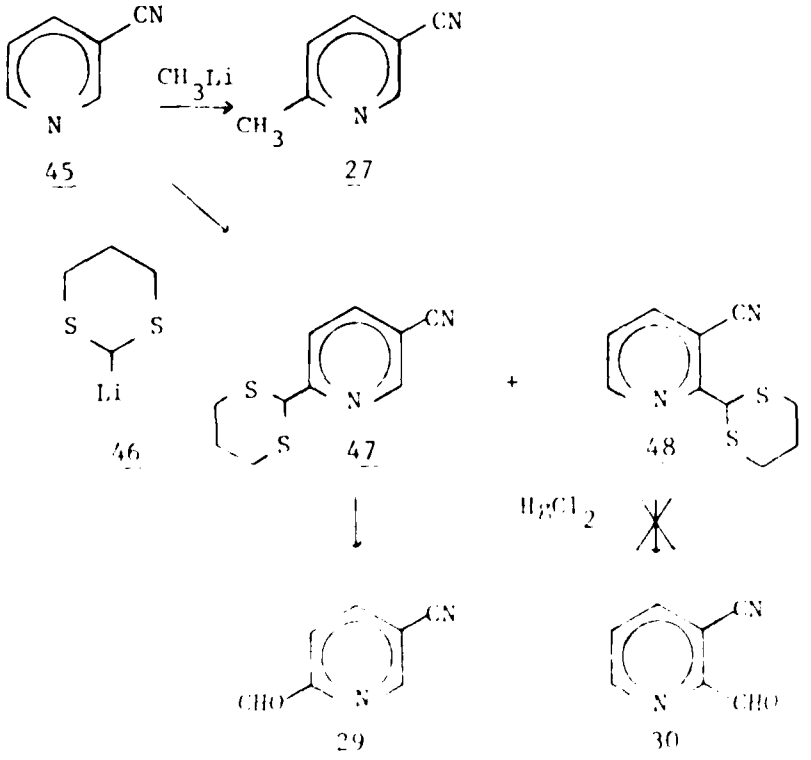
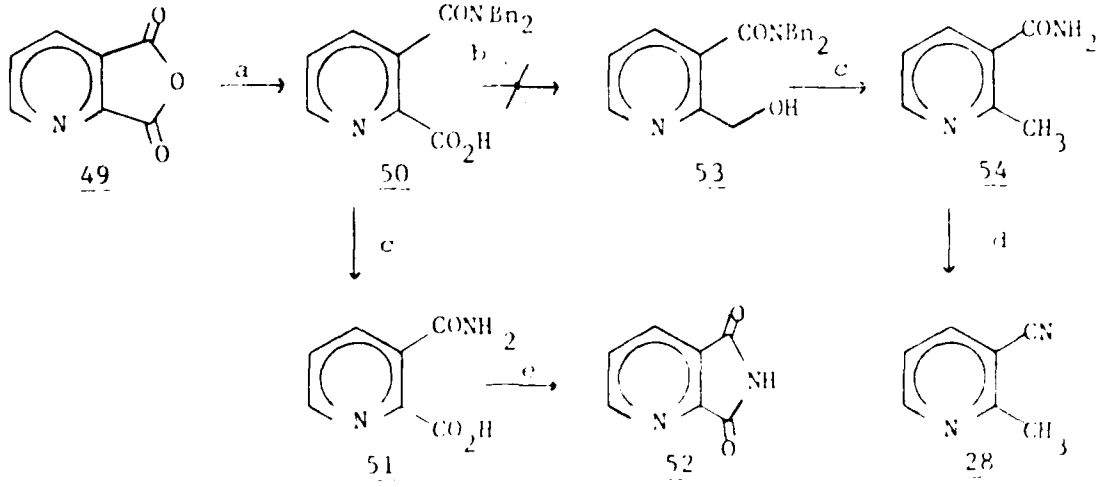


Figure 8

A separate and independent synthesis of 3-cyano-2-picoline (28) was attempted starting from 2,3-pyridinedicarboxylic anhydride 49 (Figure 9). The anhydride 49 was opened selectively with dibenzyl amine to yield 50. The benzylic protecting groups could be reduced off (hydrogen) to give the imide 52. The intent was to selectively reduce 50 to the 3-carboxamido-picoline 54, followed by conversion to the 3-cyano-2-picoline 28. The selective reduction of 50, however, could not be achieved and this route was abandoned.

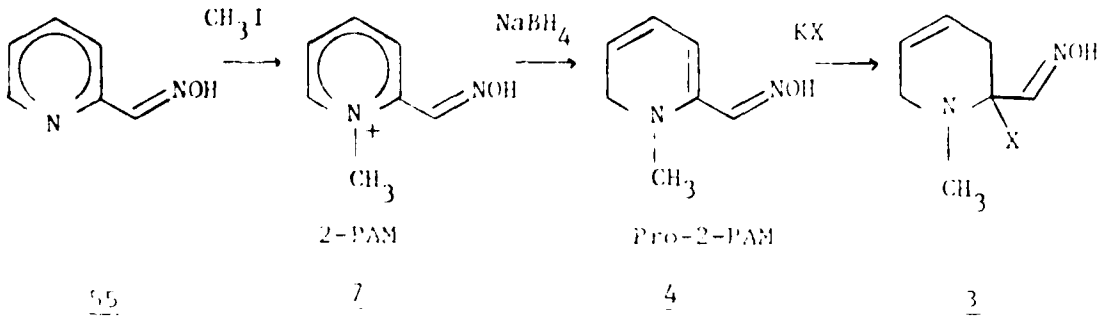


[a] $HNBn_2$. [b] $BH_3 \cdot (CH_3)_2S$. [c] H_2/cat. [d] PCl_5. [e] EtOH, r.t.

Figure 9

b. Series II - Tetrahydropyridinium Oximes

Figure 10 outlines the scheme used to synthesize the doubly latent compounds 3 (X=CN, SCN). Following the literature preparation outlined by Shek et al.[26] and starting from pyridine-2-carbaldoxime (55), the desired cyanide addition product 3 (X=CN) was obtained. The yield was considerably less than that reported by Shek et al.[26] Prodrug 3 (X=CN) was not stable at room temperature as a solid. It was reasonably stable at room temperature stored under nitrogen, but darkened considerably with time. Substitution of KSCN for KCN under identical reaction conditions (pH 1-2) did



55 7 4 3

Figure 10

not yield the desired product 3 (X=SCN). The desired product 3 (X=SCN) was isolated when the pH of the reaction was brought to the 7-8 range. A 40-60% yield was obtained and the desired product was characterized spectrally.

The cyanide addition product 3 when incubated at room temperature in pH 7.0 buffer (phosphate) was converted to a product having a λmax identical to 2-PAM.[38] Positive identification of the conversion product as 2-PAM is currently in progress.

2. Bioassay

a. In Vitro Acetylcholinesterase Assay

Regenerators of AChE were evaluated in vitro using purified AChE which was deactivated by diisopropylfluorophosphate (DFP). A convenient assay for measuring regenerated AChE activity was described by Ellman et al.[30] which utilizes acetylthiocholine as enzyme substrate and measures spectrophotometrically (412 nm) the formation of thiocholine by its reaction with dithiobis(nitrobenzoic acid) (DTNB) as illustrated in Figure 5. The major problem with using the Ellman technique in screening oxime regenerators was the fact that these regenerators caused nonenzymatic hydrolysis of acetylthiocholine at a rate which was dependent on oxime concentration (Figure 11). Evaluating the effectiveness of oxime regenerators can also be complicated by the fact that the phosphorylated oximes which form serve as potent phosphorylating agents.

To overcome these problems we modified the assay to incorporate immobilized AChE. The use of immobilized AChE had the potential advantage that regeneration of deactivated enzyme could be carried out in the absence of substrate, chromogen (DTNB) and excess DFP. Any excess oxime as well as phosphorylated oxime was washed from the system prior to measuring

Figure 11

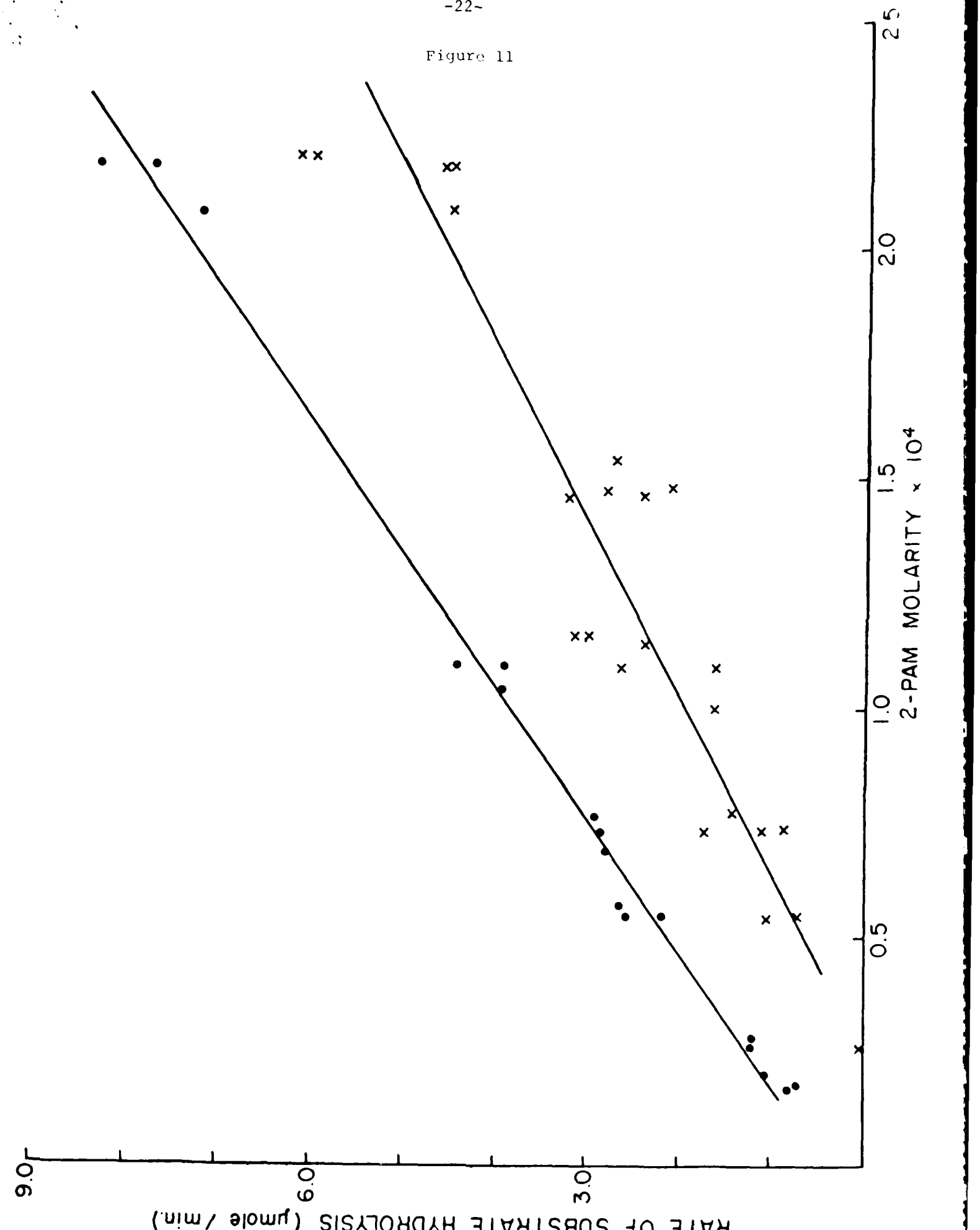

RATE OF SUBSTRATE HYDROLYSIS (μmole / min.)
9.0
6.0
3.0
0.5
1.0
1.5
2.0
2.5
2-PAM MOLARITY x 10⁴

Figure 11 Enzymatic and Nonenzymatic Hydrolysis of Acetylthiocholine

X———X, Rate of acetylthiocholine (7.5×10^{-4}M) Hydrolysis in the presence of varying concentrations of 2-PAM.

·———· Rate of acetylthiocholine (7.5×10^{-4}M) hydrolysis in the presence of varying concentrations of 2-PAM and regenerated AChE (0.15 units).

regenerated enzyme activity. Hence the inactivation, regeneration and measurement of AChE activity were carried out as separate and discrete steps.

In Figure 12 the general scheme used to immobilized AChE on polyethylene beads is shown. The process is a modification of the technique reported by Ngo, Laidler and Yam[39] for the covalent attachment of AChE to polyethylene tubing. The immobilized AChE had an activity of approximately 0.5 units/bead which was stable for up to 8 weeks stored at -16°C (MOPS buffer 0.1 M, pH 7.8, 40% glycerol). The immobilized enzyme was then incorporated into the closed loop, flow-through system illustrated in Figure 13. At low substrate concentrations the rate of AChE activity appeared to be diffusion controlled at the bead surface (Figure 14) which was consistent with the results reported by Ngo et al.[39] using AChE immobilized on polyethylene tubing.

The flow-through system allowed us in separate operations to measure AChE activity, to inactivate the enzyme with DFP, to regenerate the enzyme activity with an oxime and to measure the regenerated AChE activity. Flow rate dependent kinetics were not observed when the substrate was at saturation and in practice the assays were run at saturation with an intermediate flow rate of 5.8 ml/minute. Figures 15-17 represent the characterization of 2-PAM (7), TMB-4 (8) and MINA (10) reactivation of DFP-inactivated immobilized enzyme. In Table 1 we have compared the extent of reactivation using various reactivators as measured by others versus the method developed in our laboratory.

Table 1

Comparison of Reactivaors using Various Assays

Reactivator	Conc.	Time	% Reactivation	Method	Ref.
2-PAM	10^{-3}M	2 hr	100	Ellman	40
2-PAM	2.5×10^{-3}M	45 min	80		*
2-PAM	10^{-5}M	45 min	75	CO_2equil	41
2-PAM	2×10^{-5}M	45 min	45		*
TMB-4	10^{-5}M	45 min	100	CO_2equil	42
TMB-4	2×10^{-5}M	45 min	70		*
TMB-4	10^{-4}M	45 min	60	Titrametric	43
MINA	10^{-2}M	45 min	15	Titrametric	12
MINA	10^{-2}M	45 min	60		*

*as measured in our laboratories using immobilized AChE and the Ellman technique.

Figure 12 General Scheme for Immobilization of AChE on Polyethylene Beads

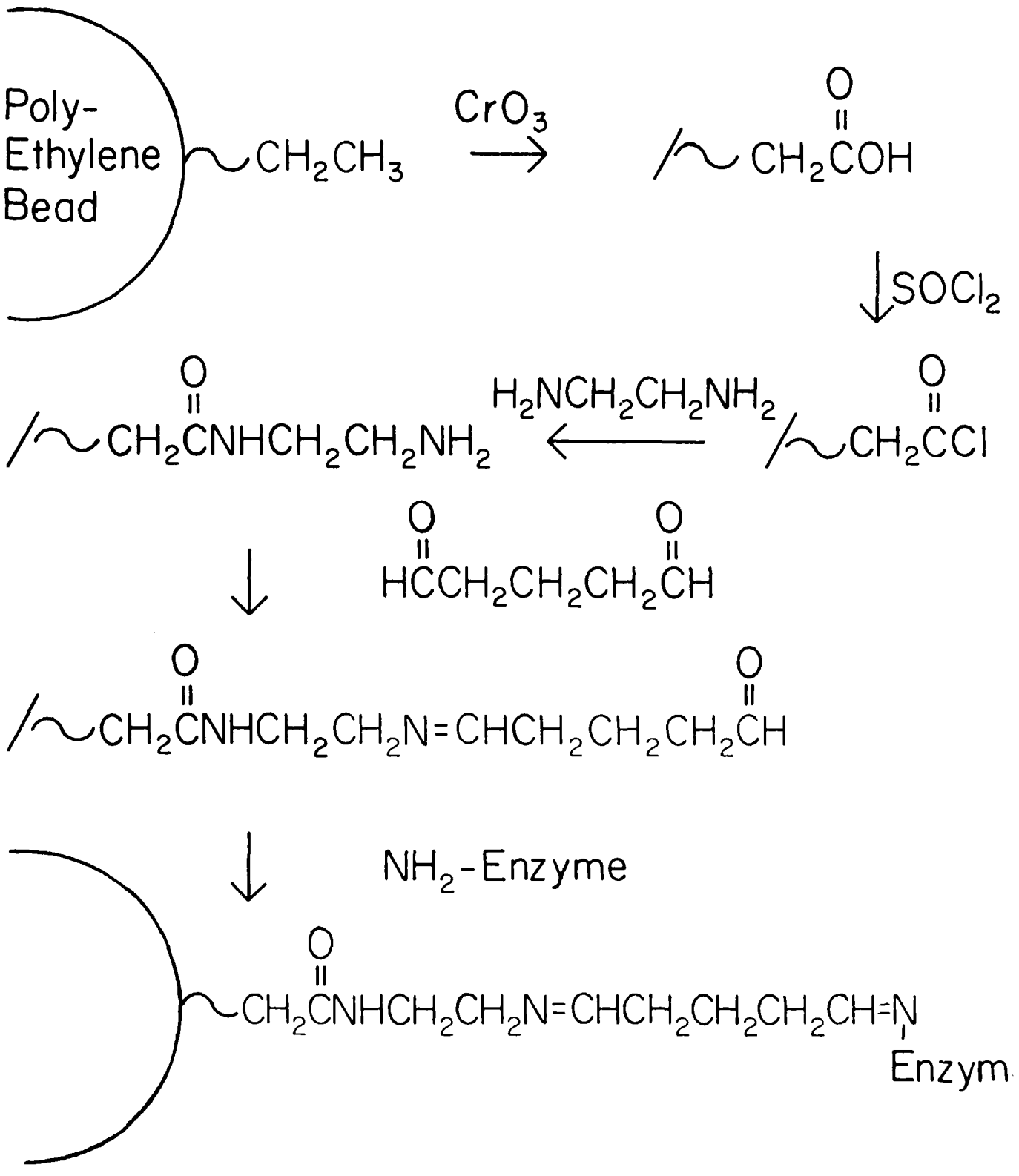

Poly-
Ethylene
Bead
CH2CH3
CrO3
CH2COH
SOCl2
CH2CCl
H2NCH2CH2NH2
CH2CNHCH2CH2NH2
HCCH2CH2CH2CH
CH2CNHCH2CH2N=CHCH2CH2CH2CH
NH2-Enzyme
CH2CNHCH2CH2N=CHCH2CH2CH2CH=N
Enzym

Figure 13 Schematic of Closed Loop, Flow-Through System for Immobilized Acetylcholinesterase Assay

Flasks 1 and 2 contained substrated (ATC, 10^{-3}M) and chromogen (DTNB, 5×10^{-4}M) in MOPS buffer (0.1 M, pH 7.8) at 37°C. The peristaltic pump was run at a flow rate of 5.8 ml/minute and the detector was set at 412 nm. The contents of Flask 1 were cycled through the column and the baseline activity of immobilized enzyme determined. DFP was then added to Flask 1 to inactivate the enzyme. The system was washed with buffer directed via switching valves. Reactivator was directed through the column for the desired exposure time, flushed from the system and the contents of Flask 2 cycled through the column to determine the level of regenerated enzyme activity.

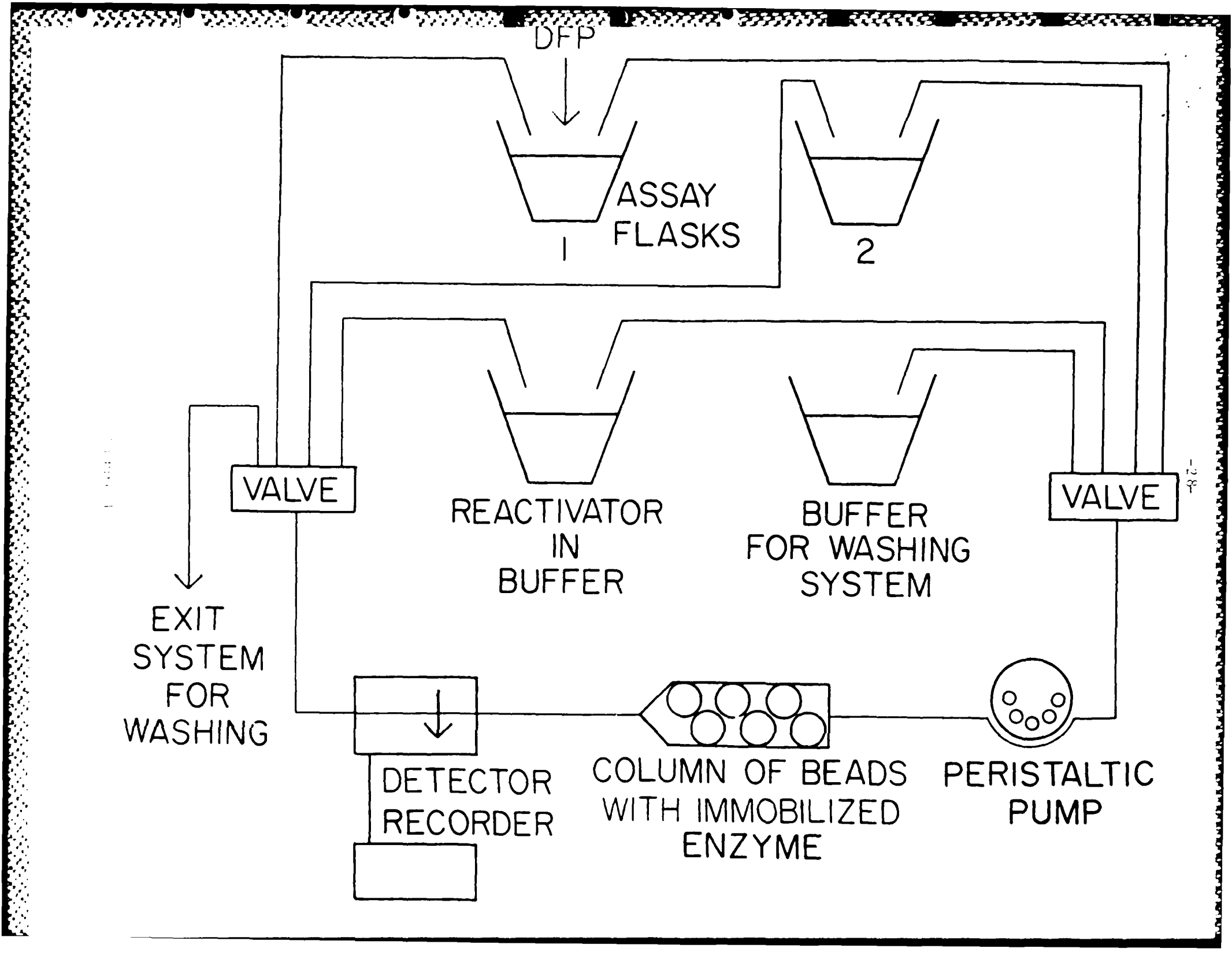

DFP
ASSAY FLASKS
1
2
VALVE
REACTIVATOR IN BUFFER
BUFFER FOR WASHING SYSTEM
VALVE
EXIT SYSTEM FOR WASHING
DETECTOR RECORDER
COLUMN OF BEADS WITH IMMOBILIZED ENZYME
PERISTALTIC PUMP

Figure 14 Lineweaver-Burke Plot of AChE Activity at Various Flow Rates of Substrate and Chromogen

A - 14 ml/min, Vmax - 4.08×10^{-6}, Km - 6.35×10^{-4};

B - 8.7 ml/min, Vmax - 4.07×10^{-6}, Km - 7.21×10^{-4};

C - 4.2 ml/min, Vmax - 4.00×10^{-6}, Km - 9.52×10^{-4}.

Figure 14

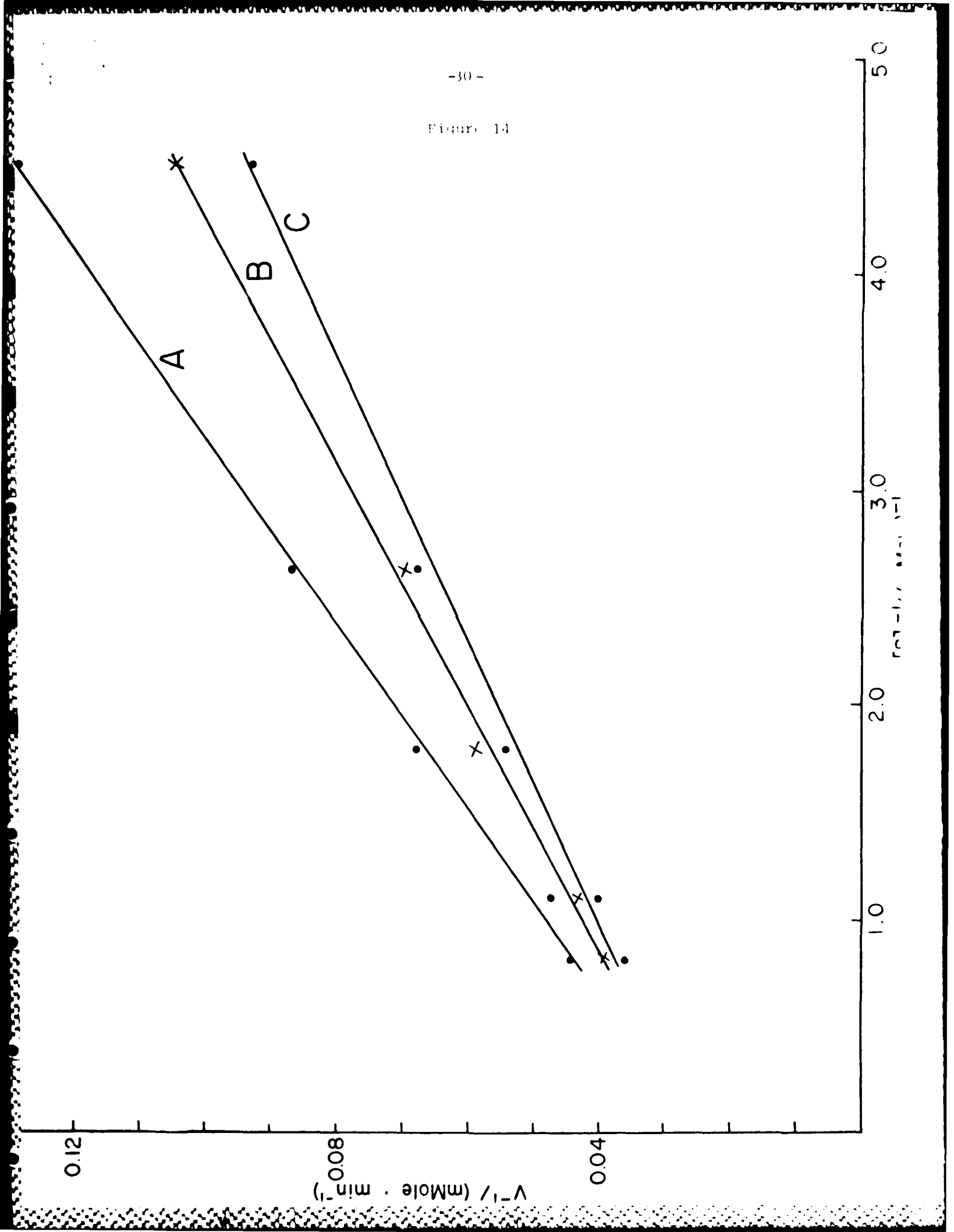


Figure 15 Effects of Varying 2-PAM Concentrations on Reactivation of Acetylcholinesterase

Concentrations expressed in molarity (M). AChE was inactivated with DFP (6×10^{-4} M). Assay conditions were as outlined in Figure 13. Treatment with varying concentrations of 2-PAM were started at time 0. At 5, 10, 20, 30 and 45 minutes, the immobilized enzyme was washed free of reactivator, then substrated and chromogen (Flask 2, Figure 13) were cycled through the system to determine the amount of regenerated enzyme activity. Following the measurement of regenerated AChE activity, exposure to regenerator was resumed.

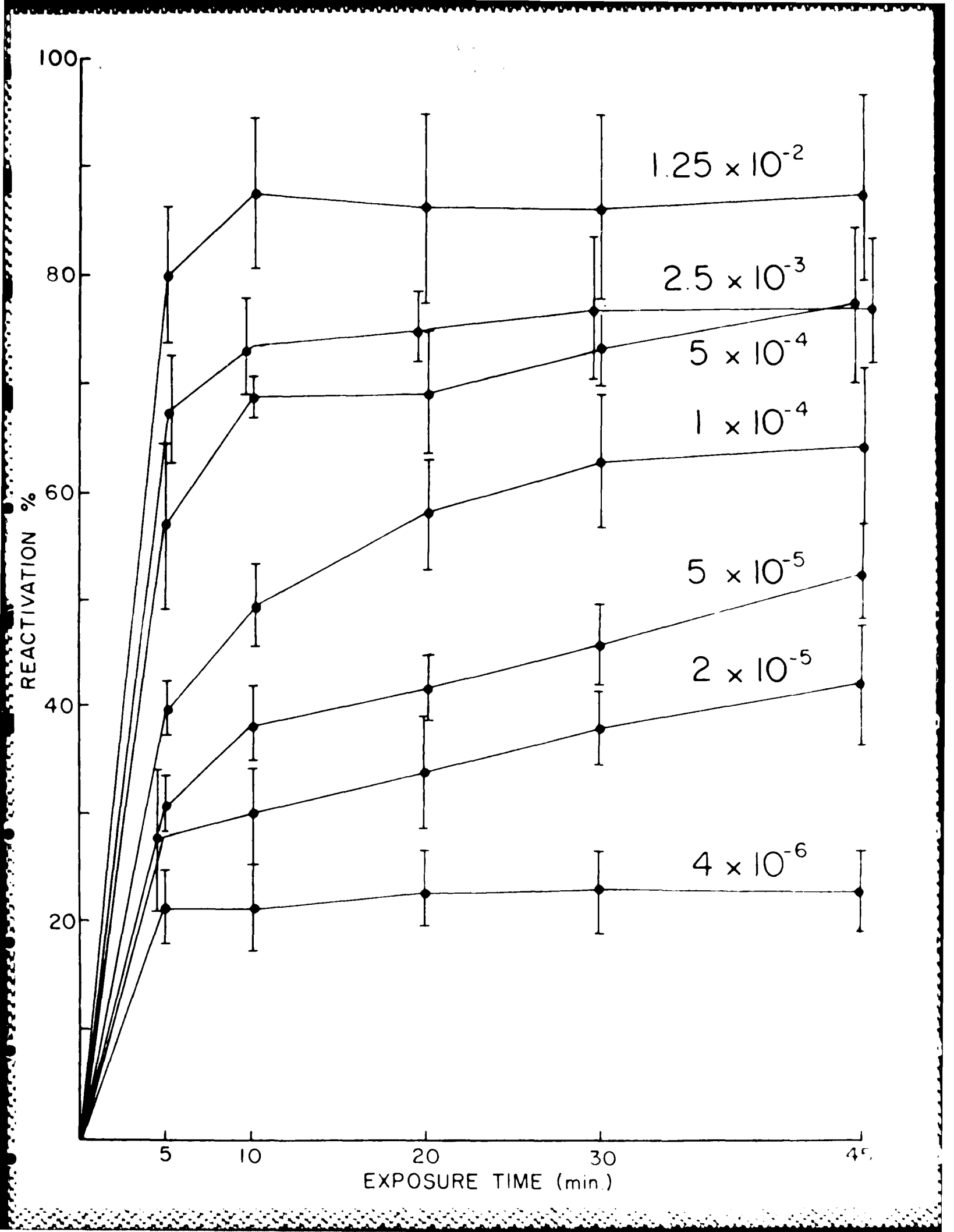

100
80
60
40
20
REACTIVATION %
1.25 x 10-2
2.5 x 10-3
5 x 10-4
1 x 10-4
5 x 10-5
2 x 10-5
4 x 10-6
5
10
20
30
EXPOSURE TIME (min.)

Figure 16. Comparison of 2-PAM and TMB-4 as Reactivators of DFP-Inactivated Acetylcholinesterase.

2-PAM and TMB-4 concentrations were [illegible] x 10[illegible] M.

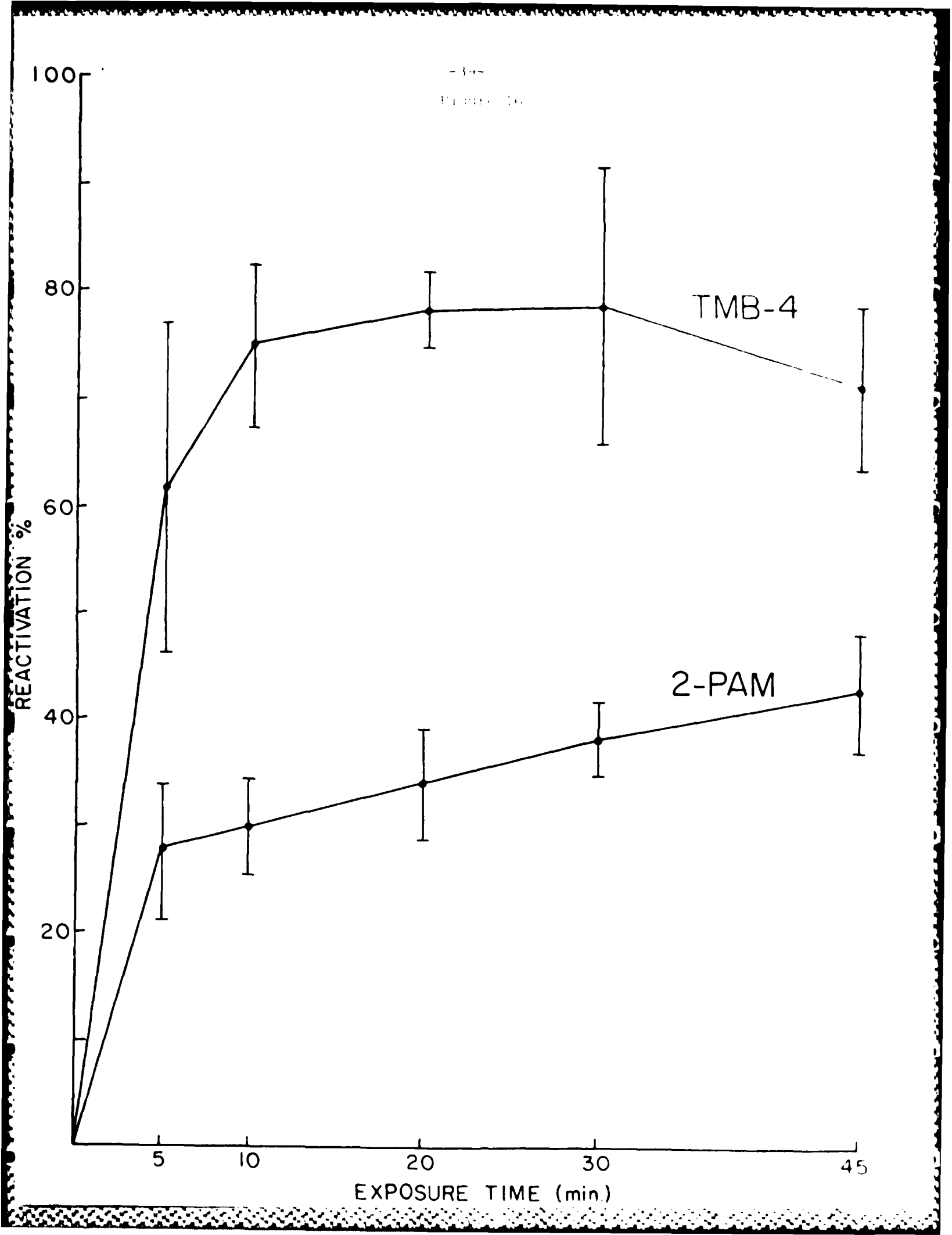
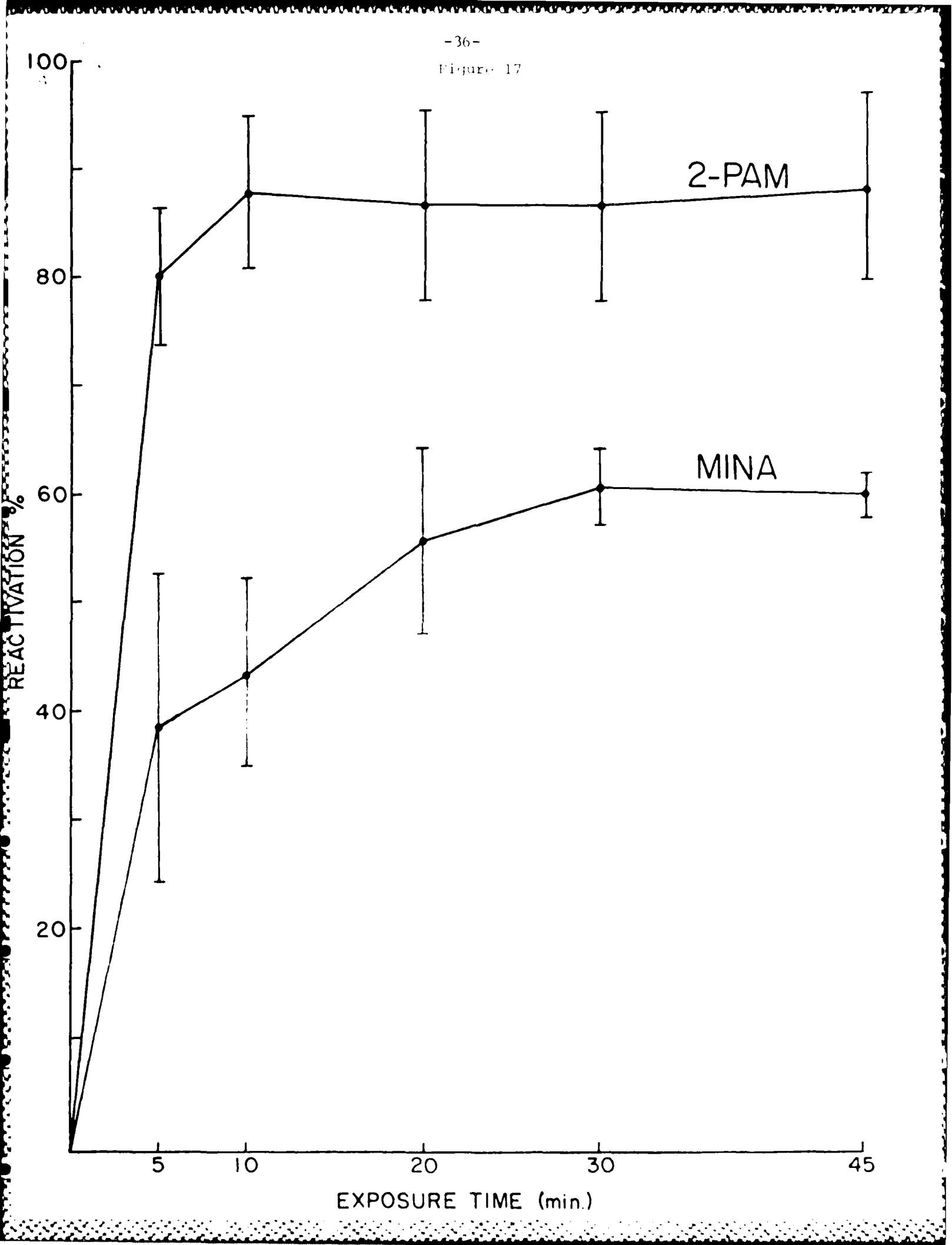

100
80
60
40
20
REACTIVATION %
TMB-4
2-PAM
5
10
20
30
45
EXPOSURE TIME (min)

Figure 17 Comparison of 2-PAM and MINA as Reactivators of DFP-Inactivated Acetylcholinesterase

2-PAM and MINA concentrations were 1.25×10^{-2} M.

Figure 17


100
80
60
40
20
REACTIVATION %
2-PAM
MINA
5
10
20
30
45
EXPOSURE TIME (min.)

E. Discussion

Some of the intended synthetic approaches have been either abandoned or revised because the procedures were cumbersome and separation & purification of the resulting products were laborious and time consuming. However, the revised synthetic schemes (Figures 7,8) developed during the past year should yield the desired 3- and 5-substituted 2-PAMs and Pro-2-PAMs. 5-Cyano-2-PAM (33) has been prepared and characterized.

Since the iodopicolines 25 and 26 were available as intermediates, we decided to synthesize the iodo-substituted 2-PAMs. Both 3- and 5-iodo-2-PAMs 41 and 42 have been prepared and characterized. The 3-iodo-2-PAM (42) has been assayed for its ability to reactivate organophosphate-inactivated AChE in vitro and it exhibits approximately 50% the activity of 2-PAM (10^{-4}M). This indicates that the active site of the enzyme can accommodate a pyridinium oxime with a large electron withdrawing substituent in the 3-position. Although a precise pka of 42 has not yet been determined, it appears that the substitution of iodine at the 3-position of 2-PAM has not significantly altered the ionization constant of the oxime.

The direct addition of a methyl equivalent to 3-cyanopyridine (45) via methyllithium provided us with an alternate and shorter route to the synthesis of 5-cyano-2-picoline (27). The addition of a "formyl equivalent" 46 to 3-cyanopyridine did afford a mixture of the 2,3- and 2,5-addition products 47 and 48. Hydrolysis of 47 afforded the 5-cyano-2-picolinealdehyde (29), but we could not successfully deprotect 48 to give 30. We were able to synthesize 3-cyano-2-picolinealdehyde 30 via CuCN displacement of 3-iodo-picolinealdehyde (38). This should provide us direct access to 3-cyano-2-PAM (34). That will allow us to draw some conclusions about steric versus electronic effects on the 2-PAM molecule when binding and reactivating capacities are examined.

It should be noted here that all of the iodo- and cyano-substituted picolines and picolinealdehydes were reasonably volatile and had low melting points. Purification of these compounds by convenient techniques such as recrystallization, distillation and sublimation was not possible.

Work on the double latentation species 3 was started only recently. However both the 2-cyano- and 2-thiocyano-tetrahydropyridinium oximes 3 (X=CN, SCN) have been prepared. It should be noted that the 2-thiocyano adduct 3 (X=SCN) required reactions conditions different from those employed in the synthesis of 3 (X=CN). It may be that for every ProPAM 3 synthesized, the nucleophilic trapping reaction will require a unqiue set of conditions.

The double latentation approach looks promising and appears to be a very exploitable approach. Since the efficacy of 2-PAM as a regenerator of organophosphate inactivated AChE has been well documented, we are not developing a new regenerator, but improving the delivery of a proven one. One potential problem with this approach is that of the lag time for conversion to 2-PAM. It may well take too long for the two step activation to occur. A latent period which is too long also puts the prodrug at the mercy of the body's metabolic system which could convert it to other forms before it can be converted into 2-PAM.

The development of the immobilized enzyme screening assay was the culmination of many frustrating attempts to make a soluble AChE assay workable. The soluble AChE assay had many drawbacks. The competition of both enzyme and regenerator for substrate was indeed discerning. The problem of excess organophosphate was a problem as well. 2-PAM is known to react directly with organophosphates in solution to generate phosphorylated 2-PAM, which is itself a potent deactivator of AChE. In addition phosphorylated 2-PAM was generated in the reactivation process. This pool of excess organophosphate and phosphorylated regenerator caused considerable problems in the soluble AChE assay.

The immobilized AChE assay system developed in our laboratory allows for the independent and sequential determination of initial enzyme activity, enzyme inactivation, enzyme regeneration and determination of regenerated enzyme activity. By washing the immobilized AChE between steps one can remove excess DFP, oxime, phosphorylated oxime, acetylthiocholine and DTNB, thus eliminating undesirable side reactions. The DFP-inactivated AChE spontaneously regenerates to about 10-15% of the initial enzyme activity. The amount of aging of the DFP-inactivated enzyme was negligible, as was the loss of enzyme activity due to enzyme degradation during the course of a two hour experiment.

We observed a great deal of variation in the activity/bead (i.e. 0.5 unit/bead) which we believe to be a function of non-uniform enzyme loading. We hope to overcome this problem by incubating the enzyme with the beads in a mechanically, slowly stirred solution and by obtaining a source of polyethylene beads which are made to uniformly tighter spherical tolerances.

The assay was effective for the determination of the regenerating capabilities of quaternary pyridinium oximes (e.g. 2-PAM and TMB-4) as well as an uncharged oxime (MINA). The order of regenerating ability was TMB-4 > 2-PAM > MINA, which was consistant with earlier observations.[12,43] There was, at all concentrations of regenerators employed, an initial rapid recovery of enzyme activity which was followed by a slower drift to higher enzyme activity. Figure 15 illustrates that the first five minutes of exposure to regenerator produces a majority of the regenerated activity observed at forty-five minutes. This phenomenon was reported earlier and was attributed to a rapid initial equilibrium followed by a slow drift to higher activity due to the breakdown of phosphorylated oxime.[44] Since our system allowed for the removal of any phosphorylated oxime, we do not feel this is a valid explanation for our experimental results. Alternative explanations could be the heterogenous nature of the enzyme itself or the effects of immobilization (i.e. multiple covalent links) which could result in a variety of different enzyme forms each exhibiting its own sensitivity to deactivation and reactivation. A comprehensive explanation of this phenomenon will require further investigation.

F. Experimental

Electron impact mass spectra were recorded on either a Varian-MAT CH-5 or Riber R-10-10 mass spectrometer with RDS data system for computer analysis and spectra printout. NMR were obtained with either a Varian T-60, Hitachi Perkin-Elmer R-24B or Varian FT-80a and were run in 1% TMS/$CDCl_3$ unless otherwise noted. The IR were obtained on either a Beckman IR-33 or AccuLab-4 spectrometer and samples were run as either neat films or KBr pellets (1:100). UV-Vis spectra were recorded on either a Cary 219 or Beckman DU-5 spectrophotometer. HPLC determinations were performed on a Beckman 342 system (112 pumps, 420 controller and 340 organizer), a Kratos 769Z variable UV detector and either a 5 mm x 15 cm Ultrasphere 5 μm ODS RP column with a 3 cm guard column or a 5 mm x 10 cm BrownLee Lichrosorb 10 μm NP column with a 3 cm guard column for separations. Melting points were obtained as either capillary melting points (uncorrected) on a Thomas-Hoover apparatus or as micromelting points (corrected) on a Fisher-Johns melting point stage. The AChE assay was equipped with a Gilson Micropuls 2 variable speed peristaltic pump and a Gilson HM Holochrome variable UV-Vis flow detector.

The chemicals and solvents were generally reagent or HPLC (Chromatography) grade unless purity was not crucial. Thin layer chromatography was carried out on Analtech SG GHLF 250 μm, Analtech Woelm SGF 1000 μm or EM SG-60 F254 200 μm plates. Liquid chromatography sorbents were either Brinkman EM SG-60 70-230 mesh or Woelm silica 63-200 mesh. The starting materials: 2-picoline, 2-pyridinecarbaldoxime and 3-cyanopyridine were obtained from Aldrich (minimum 97% purity) and were used without further purification. Acetylcholinesterase (electric eel organ) was obtained commerically purified from either Worthington (1000-1400 units/mg) or Boehringer Mannheim (1000 units/mg). Acetylthiocholine, dithiobis(nitrobenzoic acid) (DTNB), 3-[N-morpholino]propanesulfonic acid (MOPS) and diisopropylfluorophosphate (DFP) were obtained from Sigma and were used without further purification. DFP degrades slowly, even when stored at 4°C, and was replenished with a fresh supply at regular intervals to insure consistancy. The low density polyethylene beads (4mm, irregular) were supplied by Aldrich. TMB-4 was purchased from Sigma and used as received. MINA was purchased from Pfaltz and Bauer, but required purification to homogeneity by LC (SG Woelm, 8% acetone/methylene chloride v/v).

3-Iodo-2-picoline (26) and 5-Iodo-2-picoline (25)

A modification of the method of Riley and Perham was employed.[32] A 265 g (1.04 mol) protion of freshly ground iodine was dissolved in 485 ml of 30% oleum over one hour. The dropwise addition of 100g (1.07 mol) of 2-picoline to the reaction mixture via addition funnel proceeded over one hour. The reaction was brought to 150°C for 1.5 hours during which time a vigorous evolution of gas occurred. The reaction was cooled to 120°C and maintained for 12 hours. The mixture was reheated to 150°C for 3 additional hours then cooled to room temperature. The reaction was poured over 1800 ml of ice and neutralized with sodium carbonate. The neutral aqueous mixture was extracted with 2 x 500 ml of ethyl acetate. The organic extracts were washed twice with brine, dried over sodium sulfate, filtered and flashed to a black oil.

A vacuum distillation, b.p. 82-100°C at 5mm (rpt bp 108-109, 18 mm, 26, ref. 32), gave 62.3 g (27%) of 25 and 26 as a yellowish liquid. The roughly equimolar mixture of 25 and 26 could be partially resolved by LC (SG 70-230 mesh, 10% ethyl acetate/methylene chloride v/v, load ratio 1:100) and tlc (SG, ethyl ether) Rf 0.57 (26) and 0.67 (25).
26 yellow oil; NMR: δ 2.70 (s, 3H, CH_3), 6.75 (dd, 1H, Ar5H), 7.88 (dd, 1H, Ar4H), 8.38 (dd, 1H, Ar6H), $J_{4,5}$=8Hz, $J_{4,6}$=2Hz, $J_{5,6}$=5Hz (TMS/CCl_4); MS: m/e 219 (M^+), 92 (M^+-I), 66 (M^+-CN,I), 51 (M^+-CH_3, CN,I).
25 yellow-green crystals, mp (corr) 46-48°C; NMR: δ2.44 (s, 3H, CH_3), 6.88 (d, 1H, Ar3H), 7.78 (dd, 1H, Ar4H), 8.62 (d, 1H, Ar6H), $J_{3,4}$=9Hz, $J_{4,6}$=2Hz (TMS/CCl_4); MS: m/e 219 (M^+), 92 (M^+-I), 65 (M^+-HCN,I), 50 (M^+-CH_3, HCN,I).

3-Cyano-2-picoline (28) and 5-Cyano-2-picoline (27)

Using a modification of the procedure of Craig,[33] a solution containing 0.82 g (9.2 mmol) of cuprous cyanide and 2.0g (9.1 mmol) of the purified mixture 25 and 26 in 40 ml of dimethylsulfoxide was brought to a reflux for one hour. The solution was cooled to room temperature, diluted with 20 ml of water and exhaustively extracted with ethyl ether. The combined ether extracts were washed with an equal volume of brine, dried over sodium sulfate, filtered and flashed to 0.80g (75%) of yellow amorphous solid. Preparative tlc (SG, 1000 μ, ethyl ether) afforded separation of the isomers, Rf 0.48 (28) and 0.56 (27).
28 mp (corr) 48-53°C (rpt mp 58°C, 28, ref. 31); IR: (film) 2200 cm^{-1} (CN); NMR: δ 2.80 (s, 3H, CH_3), 7.27 (dd, 1H, Ar5H), 7.80 (dd, 1H, Ar4H), 8.61 (dd, 1H, Ar6H) $J_{4,5}$=8Hz, $J_{4,6}$=2Hz, $J_{5,6}$=5Hz.
27 mp (corr) 79-82°C (rpt mp 84-85°C, 27, ref. 45). IR: (KBr) 2200 cm^{-1} (CN); NMR: δ 2.66 (s, 3H, CH_3), 7.28 (d, 1H, Ar3H), 7.85 (dd, 1H, Ar4H), 8.78 (d, 1H, Ar6H), $J_{3,4}$=8Hz, $J_{4,6}$=2Hz; MS: m/e 118 (M^+), 103 (M^+-CH_3), 91 (M^+-HCN), 76 (M^+-HCN, CH_3).

5-Cyano-2-picoline (27)

The low temperature addition technique of Parks et al.[46] was used. A 2.0 g (19 mmol) portion of 3-cyanopicoline (45) was dissolved in 20 ml THF and cooled to -40°C (solubility limit). Under a nitrogen atmosphere 13.7 ml (21 mmol, 1.55M in ether) of methyllithium was added dropwise at a rate which maintained the reaction temperature between -30 and -40°C. The deep red-orange mixture developed a heavy precipitate which redissolved on slow warming to room temperature. After a total reaction time of two hours, the reaction was quenched with 20 ml of water and vigorous stirring. The mixture was acidified to pH 2-3 with aqueous hydrochloric acid and extracted with 2 x 10 ml of ether. The ether extracts were washed with brine, dried over sodium sulfate, filtered and flashed to 0.60 g (26%) of crude 27 as a light yellow-orange oil. Sublimation (40°C, 0.05 mm) afforded colorless crystals of 27. If the acidic aqueous layer was adjusted to pH 7-8 and extracted, 1.0 g (36%) of 3-acetylpyridine was isolated. Physical and spectral data reported in synthesis of 27 and 28 (experimental).

3-Iodo-2-picolinealdehyde (38) and 5-Iodo-2-picolinealdehyde (37)

Employing a modification of the method of Markovac et al.[15], 11.9 g (54 mmol) of purified 25 and 26 was mixed with 13.3 g (52 mmol) of iodine. A solid picoline-iodine complex formed which was gently melted, stirred to homogeneity and allowed to resolidify. The cake was broken up and dissolved in 25 ml of dimethylsulfoxide. The solution was added dropwise via an addition funnel to 30 ml of dimethylsulfoxide which was preheated to 150°C. The reaction mixture was maintained at 150-160°C for 40 minutes during which time evolving dimethylsulfide was trapped in a 20% perchloric acid bubbler. The reaction was cooled to room temperature, neutralized with a saturated sodium bicarbonate solution and extracted exhaustively with ethyl ether. The combined ether extracts were washed with brine, dried over sodium sulfate, filtered and flashed to 13 g of a viscous black tar. The entire crude was loaded onto a silica gel column (70-230 mesh, load ratio 1:80). Undesired materials were eluted off with methylene chloride (approx. 2L). The desired aldehydes 37 and 38 were eluted off by a 0-25% ethyl acetate/methylene chloride v/v gradient. The ethyl acetate concentration was increased 5% in 500 ml increments. The overall yield was 8.7 g (69%) of which 3.2 g (25%) was pure 5-iodo isomer 37, 3.5 g (28%) pure 3-iodoaldehyde 38 and the remainder a mixture of isomers; tlc (SG, 10% ethyl acetate/methylene chloride v/v) Rf 0.48 (38) and 0.65 (37). Yields ranged from 30-69%.
38 - needles, pleasant minty order, mp (uncorr) 66-68°C; IR: (KBr) 2860 cm^{-1} (CHO), 1700 (CO); NMR: δ 7.15 (dd, 1H, Ar5H), 8.32 (dd, 1H, Ar4H), 8.73 (dd, 1H, Ar6H), 9.83 (s, 1H, CHO), $J_{4,5}$=8 Hz, $J_{4,6}$=2 Hz, $J_{5,6}$=5 Hz (1% TMS/CCl_4); MS: m/e 233 (M^+), 205 (M^+-CO), 78 (M^+-CO, I).
37 - mp (corr) 109-111°C; IR: (KBr) 2840 cm^{-1} (CHO), 1695 (CO); NMR: δ 7.67 (d, 1H, Ar3H), 8.18 (dd, 1H, Ar4H), 8.98 (d, 1H, Ar6H), 9.96 (s, 1H, CHO), $J_{3,4}$=8Hz, $J_{4,6}$=2Hz (1% TMS/CCl_4); MS: m/e 233 (M^+), 205 (M^+-CO), 204 (M^+-CHO), 149 (M^+-CO,I, HCN).

5-Cyano-2-picolinealdehyde (29)

The preparation of this compound followed the procedure as described for the 3- and 5-iodopicolinealdehydes 37 and 38. Starting with 0.80 g (6.8 mmol) crude 5-cyanopicoline (27), 0.31 g (36%) of 29 was isolated as pale yellow needles. Yields ranged from 10-36%. The aldehyde could be purified by sublimation (55°C at 15 mm). IR: (KBr) 2860 cm^{-1} (CHO), 2225 (CN), 1710 (CO); NMR: δ 8.04 (m, 2H, Ar 3,4H), 9.01 (d, 1H, Ar6H), 10.07 (s, 1H, CHO), $J_{4,6}$=2Hz; MS: m/e 132 (M^+), 104 (M^+-CO), 103 (M^+-CHO), 77 (M^+-CO, HCN).

3-Cyano-2-picolinealdehyde (30)

A modification of the method of Craig was used.[13] A slurry of 0.250 g (1.0 mmol) of 3-iodopicolinealdehyde (38) and 0.192 g (2.2 mmol) of cuprous cyanide in 10 ml of dry dimethylsulfoxide was heated to 170°C for five hours with vigorous stirring. The reaction was cooled to room temperature and stirred overnite. The mixture was extracted exhaustively with ethyl ether and the ether extracts washed with aqueous hydrochloric acid (pH 3-4), saturated brine, dried over sodium sulfate, filtered and flashed to a residue. The product was purified via tlc (SG GF, 1000 microns, 5% ethyl

acetate/methylene chloride v/v) to give 0.020 g (14%) pale yellow oil 30. HPLC (NP, 10 cm + 3 cm guard column, 50% ethyl acetate/hexane, 1 ml/min, 270 nm) showed 30 had a retention time of 3.3 min (minor impurities at 2.3 and 4.1 min) versus 3.1 min for 38. 30 - IR: (neat) 2850 cm^{-1} (CHO), 2200 (CN), 1725 (CO).

2-(5-Cyano-2-pyridinyl)-1,3-dithiane (47)

Method A

Using a modification of the method of Corey and Seebach,[47] 0.64 g (5.3 mmol) of 1,3 dithane was dissolved in 10 ml of dry THF. The solution was cooled to -40°C and, under nitrogen, 2.2 ml (5.8 mmol, 2.6 M in hexane) of butLi was added dropwise. The reaction was maintained at -40°C for 90 minutes then transferred, dropwise under nitrogen pressure via a cannula to a solution of 0.50 g (4.8 mmol) of 3-cyanopyridine in 30 ml of dry THF. The addition of the dithiane anion to 3-cyanopyridine proceeded over thirty minutes. The addition reaction mixture was maintained at -30°C for approximately two hours and warmed slowly to room temperature over an additional two hours. The reaction was quenched with 20 ml of water and vigorous stirring while maintaining the temperature below 35°C. The organic layer was separated and the aqueous layer extracted with 2 x 15 ml portions of ethyl acetate. The combined organic layers were washed with brine, dried over sodium sulfate, filtered and flashed to 1.14 g of crude product as a viscous tan oil. Purification by column chromatography (Woelm SG 60, 63-200 mesh, load ratio 1:500, methylene chloride) afforded 0.16g (18%) of 47. TLC (E. Merck, methylene chloride) Rf 0.27.
47 - light tan needles, mp 117-121°C; IR: (KBr) 2210 cm^{-1} (CN); NMR: 2.15 (m, 2H, $SCH_2\underline{CH_2}$), 3.05 (m, 4H, $SC\underline{H_2}$), 5.35 (s, 1H, SCHS), 7.63 (d, 1H, Ar3H), 8.00 (dd, 1H, Ar4H), 8.35 (d, 1H, Ar6H); $J_{3,4}$=8Hz, $J_{4,6}$=2Hz; MS: m/e 222 (M^+), 189 (M^+-SH), 104 (M^+-1,3-dithiane).

2-(3-Cyano-2-pyridinyl)-1,3-dithane (48) and
2-(5-Cyano-2-pyridinyl)-1,3-dithiane (47).

Method B

A 1.0 g (8.3 mmol) portion of 1,3-dithiane was dissolved in 20 ml dry THF and chilled to -70°C under nitrogen. Via syringe 5.3 ml (8.3 mmol, 1.6 M in hexane) butLi was added over three minutes. The golden solution was maintained at -70°C for 90 minutes with stirring and then a solution of 0.866 g (8.3 mmol) of 3-cyanopyridine (45) in 10 ml dry THF was added dropwise over 10 minutes. The light reddish solution was maintained at -10°C, under nitrogen, with vigorous stirring for approximately two hours, then warmed slowly to room temperature. The reaction was quenched with 20 ml of water maintaining the temperature below 35°C. The mixture was acidified to pH 2-3 with aqueous hydrochloric acid. The organic layer was separated and the aqueous phase extracted with 2 x 50 ml portions of methylene chloride. The combined organic extracts were washed with 2 x 50 ml brine, dried over sodium sulfate, filtered and evaporated to 1.75 of crude material. The material was purified by LC (Woelm SG, 63-200 mesh, 5% ethyl acetate/methylene chloride v/v, load ratio 1:135). Work up of the column fractions afforded 0.111 g

(6%) 47 and 0.083 g (4.5%) 48. (Note - if 45 was added to the dithiane anion at -10°C, up to 55% yields of almost exclusively the 2,3-isomer 47 could be obtained.) HPLC (NP, 10 cm + 3 cm guard column, 40% ethyl acetate/hexane, 1 ml/min, 265 nm) showed retention times 2.64 min (47) and 3.24 min (48) versus 6.2 min (45).
47 - see method A experimental for physical and spectral properties.
48 - light tan needles, mp 96-102; IR: (melt) 2220 cm^{-1} (CN); NMR: δ 2.13 (m, 2H, $SCH_2\underline{CH}_2$), 3.04 (m, 4H, $SC\underline{H}_2$), 5.47 (s, 1H, SCHS), 7.72 (d, 1H, Ar5H), 8.81 (d, 1H, Ar4H), 8.86 (s, 1H, Ar6H), $J_{4,5}$=5Hz; MS: m/e 222 (M^+).

5-Cyano-2-picolinealdehyde 29

The basic method of Seebach[35] was used. A 0.102 g (0.45 mmol) portion of 47 and 0.097 g (0.45 mmol) of mercuric oxide were stirred under nitrogen in 2 ml of 10% water/methanol v/v. To this was added a solution of 0.244 g (0.9 mmol) mercuric chloride in 1 ml 10% water/methanol v/v dropwise. After approximately five minutes the reaction was brought to a gentle reflux and maintained for approximately four hours. The reaction was cooled to room temperature, the insoluble material filtered off and rinsed with methylene chloride. The filtrate was reduced to a smaller volume, washed with brine, filtered through a cotton plug and evaporated to a 0.060 g (quant) of a colorless solid. Sublimation (40-60°C , 0.05-0.1 mm) afforded colorless crystals identical to those synthesized via oxidation of 5-cyanopicoline (27) (see earlier experimental).

3-Iodo-2-pyridinealdoxime (40)

A saturated aqueous solution of 10.015 g (0.21 mmol) hydroxylamine hydrochloride was neutralized with sodium carbonate and added to a methanolic solution of 0.021 g (0.09 mmol) of aldehyde 38. Within a short time a fine precipitate was evident. The solution was warmed gently to boiling and allowed to cool with stirring overnight. The white needles were suction filtered, the supernate concentrated and a second crop of product collected. The combined crops afforded 0.019 g (82%) of oxime 40. Yields ranged from 42-82%.
40 - mp 235-237 (corr); IR: (KBr) 3400 cm^{-1} (brd, OH), 1610 (CH=N); NMR: δ 7.15 (dd, 1H, Ar5H), 8.34 (dd, 1H, Ar4H), 8.61 (m, 2H, Ar6H and CHNOH), $J_{4,5}$=8Hz, $J_{4,6}$=2Hz, $J_{5,6}$=5Hz (TMS/$CDCl_3$/CD_3SOCD_3); MS: m/e 248 (M^+), 205 (M^+-CHNO), 204 (M^+-CHNOH), 104 (M^+-I, OH), 91 (M^+-NO, I).

5-Iodo-2-pyridinealdoxime (39)

Preparation of 39 was as described for 40. A 0.73 g (3.1 mmol) portion of 37 was reacted with 0.65 g (9.3 mmol) of hydroxylamine hydrochloride to give 0.69 g (89%) of 39.
39 - colorless needles mp (corr) 211-214°C; IR: (KBr) 3400 cm^{-1} (brd, OH), 1560 (CH=N); NMR: δ 7.64 (d, 1H, Ar3H), 8.00 (dd, 1H, Ar4H), 8.12 (s, 1H, CHNOH), 8.79 (d, 1H, Ar6H), $J_{3,4}$=9Hz, $J_{4,6}$=2Hz (TMS/$CDCl_3$/CD_3SOCD_3); MS: m/e 248 (M^+), 204 (M^+-CHNOH), 121 (M^+-I), 104 (M^+-I,OH), 94 (M^+-I,HCN), 91 (M^+-I,NO).

5-Cyano-2-pyridine carbaldoxime (31)

Preparation of 31 was as described for 40. A 0.157 g (1.19 mmol) portion of crude 29 was reacted with 0.082 g (1.19 mmol) of hydroxylamine hydrochloride to give 0.097 g (55%) of 31 - colorless needles, mp (uncorr) 190-200°C; IR (KBr) 2220 cm^{-1} (CN), 1600 (CH=N); NMR: δ 8.10 (m, 3H, Ar 3, 4H and CHNOH), 8.93 (d, 1H, Ar6H) 11.81 (s, 1H, CHNOH), (TMS/$CDCl_3$/CD_3SOCD_3); MS: m/e 147 (M^+), 129 (M^+-H_2O), 117 (M^+-NO), 104 (M^+-CHNO), 90 (M^+-NO,HCN), 76 (M^+-HCN, CHNOH).

3-Iodo-2-pyridine carbaldoxime Methiodide (42)

A modification of the procedure of Poziomek, Hackley and Steinberg was used.[48] A 0.029 g (0.12 mmol) portion of oxime 40 was dissolved in 2 ml absolute ethanol along with 150 µl (2.4 mmol) of methyl iodide. The reaction was carried out in an ampule which was purged with nitrogen prior to sealing. The ample was heated to 95°C in a sand bath for 16 hours. Upon cooling a bright yellow solid crystallized out of the reaction mixture. Suction filtration resulted in the isolation of 0.014 g (33%) of the desired pyridinium salt 42

42 - yellow needles, mp (corr) 195-205°C (dec); IR: (KBr) 3200 cm^{-1} (brd OH), 1620 (CH=N); NMR: δ 4.31 (s, 3H, CH_3), 7.68 (dd, 1H, Ar5H), 8.41 (s, 1H, CHNOH), 8.83 (d, 1H, Ar6H), 8.96 (d, 1H, Ar4H), $J_{4,5}$=8Hz, $J_{5,6}$=6Hz (D_2O).

5-Iodo-2-pyridine carbaldoxime methiodide (41)

Preparation of 41 was as described for 42. Starting with 0.05 g (0.2 mmol) of 39 and 0.35 ml (5.6 mmol) of methyl iodide the reaction was run for 2 days at 95°C. A bright yellow crystalline solid 41, 0.006 g (8%) was isolated.

41 - mp (corr) 237°C (dec); IR: (KBr) 3150 cm^{-1} (brd OH), 1620 (C=N).

5-Cyano-2-pyridine carbaldoxime methiodide (33)

Preparation of 33 was as described for 42. Starting with 0.075 g (0.51 mmol) of 31 and 1.1 ml (17.7 mmol) of methyl iodide the reaction was run at 100°C for 7 hours. A second 1.1 ml portion of methyl iodide was added and the reaction continued for an additional 6 hours. The crude product 33 was loaded onto a Dowex-50W ion exchange resin and eluted off with 1N hydrochloric acid (35% water/methanol v/v) to afford 0.010 g (7%) 33. IR: (KBr) 3100 cm^{-1} (v.brd, OH), 2200 (v.weak, CN), 1640 (CH=N); NMR δ 4.34 (s, 3H, CH_3) 8.19 (s, 1H), 8.38 (d, 1H), 8.89 (d, 1H), 9.35 (d, 1H), (D_2O).

2-Pyridine carbaldoxime Methiodide (7)

The method of Poziomek, Hackley and Steinberg was used.[48] A 20 g (0.16 mol) portion of 2-pyridinealdoxime (55) and 31 ml (0.33 mol) methyl iodide were brought to a gentle reflux in 150 ml 95% ethanol for 24 hours. Upon cooling brilliant faint yellow crystals precipitated out of solution. The crystals were isolated by suction filtration, washed with portions of absolute ethanol and vacuum dried to afford crude product. Two recrystallizations from ethanol gave 33.3 g (77%) of the desired 2-PAM (7), mp (corr) 222°C (dec) (rpt 220°C, ref. 4).

N-Methyl-2-cyano-1,2,3,6-tetrahydropyridine-2-carbaldoxime (3, X=CN)

The procedure of Bodor, Shek and Higuchi[26] was employed starting with 2.5 g (9.5 mmol) of 2-PAM (7), 0.53 g (38%) of 3 (X=CN) was isolated as a light tan solid. The material darkened considerably with time when stored at room temperature in air. Even when stored under nitrogen the compound was not especially stable. 3(X=CN), mp 101-104°C (rpt 112-114, ref. 26); IR: (KBr) 3200 cm^{-1}(brd OH), 2300 (CN), 1650 (C=N); NMR: δ 2.38 (s, 3H, CH_3), 3.15 (m, 4H, CH_2CH), 5.75 (m, 2H, $CH_2CHCHCH_2$), 7.35 (s, 1H, CHNOH), (TMS/CD_3COCD_3).

N-Methyl-1,2,3,6-tetrahydropyridine-2-carbaldoxime-2-thiocyanate (3 X=SCN)

A modification of the method of Bodor, Shek and Higuchi was used.[26] Into 10 ml of water was dissolved 1.5 g (15.4 mmol) of potassium thiocyanate and 1.0 g (3.8 mmol) of 2-PAM (7). The solution was degassed with nitrogen, chilled to 0°C and the pH adjusted to 1-2 with concentrated hydrochloric acid. The aqueous solution was layered with 50 ml of ethyl ether and 0.2 g (5.3 mmol) of sodium borohydride was added in one protion. The temperature was maintained at 0°C until the ebullition ceased and then the reaction was allowed to warm to room temperature slowly. The nitrogen atmosphere was maintained and the pH monitored throughout the reaction. The pH leveled off at 7-8 and remained at that figure through most of the reaction. After four hours the aqueous layer was saturated with sodium chloride, the ether layer removed and the aqueous layer extracted with a second 50 ml portion of ether. The combined ether extracts were washed with brine, dried over sodium sulfate, filtered and evaporated to 0.240 g (32%) of a light pink solid 3 (X=SCN). The product darkened considerably with time even when stored under a nitrogen atmosphere. 3 (X=SCN) - mp (corr) 94-96°C; IR (KBr) 2110 cm^{-1} (SCN), 1665 (HC=N); NMR: δ 2.35 (s, 3H, CH_3), 3.20 (m, 4H, $CH_2CHCHCH_2$), 5.70 (m, 2H, CH_2CHCH), 7.45 (d, 1H, CHNOH), (TMS/CD_3COCD_3).

Immobilization of Acetylcholinesterase

The immobilization process was a modification of the technique according to Ngo et al. see figure 12).[39] The modifications in the procedure for functionalization of the polyethylene bead surface were as follows: the reactions with thionylchloride, ethylenediamine and rinses were carried out under an anhydrous nitrogen atmosphere. Treatment with glutaraldehyde was extended for three hours with one solution change. MOPS buffer (100 mM, pH 7.8) was used instead of phosphate buffer. The enzyme was fastened to the bead surface in a two hour room temperature incubation. The beads containing immobilized enzyme were stored at -16°C in MOPS buffer (above) prepared in 40% glycerin. The enzyme was damaged by repeated freeze thaw cycles and was, therefore, stored in small batches. The activity was stable for at least two months. The activity per bead was approximately 0.5 units/bead, however, there were wide variations from bead to bead. Under typical assay conditions (MOPS buffer, 37°C) the enzyme activity declined 1-2%/hr.

Assay

The enzyme activity was measured as outlined in the Ellman technique (see Figure 5).[30] The substrate (acetylthiocholine) was dissolved in 90% ethanol and chromogen (DTNB) in 95% ethanol prior to dilution in 30 ml MOPS buffer to give concentrations of 10^{-3} M and 5×10^{-4} M respectively. Substrate concentration was at saturation level as determined from kinetics data.

The closed loop flow through system (Figure 13) allowed changing of flasks through the use of small switching valves. The flow rate was routinely set at 5.8 ml/min. The columns containing immobilized enzyme normally consisted of 6-8 polyethylene beads (3-4 units) with glass bead spacers. Continuous monitoring of column effluent was performed with a flow cell in the detector set at 412 nm. The recorder plotted absorbance units versus time and was converted to rate of product formation using the conversion outlined by Ellman.[30] Data points in Figures 15-17 represent a minimum of three replications.

The baseline enzyme activity was established, then an excess of DFP (6×10^{-4}M) was introduced. Total inactivation was confirmed by a stable flat baseline (approx. 5 min). The excess DFP was washed away with buffer. Reactivators were routinely pumped through the enzyme containing column in a single pass fashion for the first five minutes to ensure removal of phosphorylated oxime. During the remaining time the solution could be recycled. The extent of enzyme regeneration at 45 minutes was the same regardless of whether the regenerator was recycled after the first five minutes or pumped through in a continuous single pass fashion. At 5, 10, 20, 30 and 45 minutes the immobilized enzyme was washed free of regenerator, then substrate and chromogen (Flask 2, Figure 13) were cycled through the system to determine the amount of regenerated enzyme activity. Following the measurement of regenerated AChE activity, exposure to regenerator was resumed.

The concentration of reactivator was monitored before and after regeneration via HPLC to insure no loss of reactivator due to absorption or specific binding to beads etc. Using an isocratic system of 80% acetonitrile/0.01 M heptane sulfonic acid adjusted to pH 3.5 with acetic acid and monitoring at 265 nm, the reproducible limits of detection were approximately 100 pg. Similar results have been reported in the literature.[49, 50]

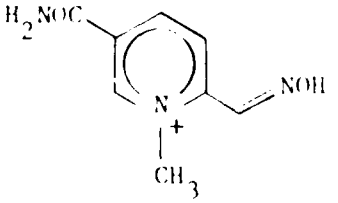
G. Recommendations

1. Chemistry

Our priorities are as follows:

a) Large scale synthesis (2-4 g) of 3/5-cyano- and 3/5-iodo-2-PAMs 33, 34, 41 and 42 (Figure 7). Collect elemental analysis for all new compounds and mass spectral data for all non-pyrolyzable new compounds. In vitro screening for AChE regenerating activity should provide sufficient information to choose one or all of these compounds for survival studies in animals treated with lethal doses of DFP. If parent quaternary compounds are sufficiently active the dihydro- or Prodrug form will be synthesized and screened in vivo.

b) Convert the 3- and 5-cyanopicolinealdehyde intermediates 29 and 30 to their 3/5-carboxamides and subsequent syntheses of carboxamide substituted 2-PAMs 56 and 57. These 3/5-carboxamides 56 and 57 will be screened as above and the dihydro-Prodrug forms made as required.



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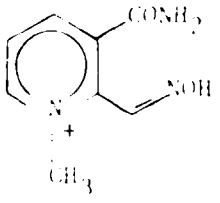

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c) Continue investigations in the double latentation approach (Figure 10). Several analogs of 3 (Figure 9) where -X is "ideal" (i.e. X is relatively non-toxic, labile enough to eliminate at physiologic pHs and nucleophilic enough to form the desired tetrahydro-addition product) will be synthesized. We will examine closely the conversion rates to 2-PAM in vitro in a pH window of 6-8 monitoring not only the appearance of 2-PAM, but any nonquaternary decomposition products. The most favorable candidates will then be tested in whole animal survival studies.

d) The pKa's for both prodrug and parent quaternary forms will be determined as well as their octanol/buffer partition coefficients.

2. Biology

a) We plan to further investigate the observed phenomenon of the dependence of extent of reactivation of immobilized AChE on the concentration of reactivator and subsequently determine a plausible mechanism for this phenomenon.

b) Initiate animal survival studies of the most active (from in vitro testing) 3/5-substituted-2PAMs, their prodrug forms (Series I) and the tetrahydropyridinium oximes (series II).

c) Initiate tissue distribution studies for the most active parent quaternary pyridinium oximes and their prodrug forms.

d) Initiate CNS AChE activity determinations in mice - for organophosphate poisoned animals and those "saved" by both parent quaternary oximes and prodrug forms.

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Appendix A

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SIGNIFICANT DIFFERENCES IN SOMAN INDUCED INHIBITION AND RECOVERY OF ACETYLCHOLINESTERASE IN BRAIN, PERIPHERAL NERVE AND MUSCLE OF RAT. Wolf-D. Dettbarn. Vanderbilt Univ., Nashville, TN 37212

Inhibition of AChE activity was studied in nerve tissue and muscle after subcutaneous injection of 0.060 mg/kg Soman (O-11,2,2-trimethylpropyl/methylphosphonofluoridate). Following 60 min after drug application, AChE inhibition was greatest in brain (B) with 6% of enzyme activity remaining, while enzyme activity in soleus (S), extensor digitorum longus (EDL) and diaphragm (D) was reduced to 25%. AChE of peripheral nerve (PN) showed the lowest sensitivity to soman, enzyme activity was only reduced to 76% of control. Recovery was fastest in PN, since within 24 hours following soman enzyme activity had returned to normal. All other tissues tested showed slow recovery during the first two days. Based on the reappearance of AChE and assuming first order kinetics, the following half-life values in days were calculated; Brain 12.5, Soleus 5.7, EDL 9.0, Diaphragm 9.0. Only low molecular weight forms such as 10S and 4S recovered during the first two days and no activity of the 16S form was observed, in spite of obvious neuromuscular transmission. By day seven, the 4S form had completely recovered, while the 16S form had only returned to 50%. Slow attachment of the AChE into subcellular structure may explain the apparent slow recovery of the 16S forms. This work was supported by Air Force Grant #82-0310.

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AN EFFICIENT IN VITRO ASSAY FOR ACETYLCHOLINESTERASE REGENERATORS USING IMMOBILIZED ENZYME. J. E. Simmons,* A. M. Trammel* and R. T. Borchardt. The Center for Biomedical Research, Univ. of Kansas, Lawrence, KS 66044.

An improved acetylcholinesterase (AChE, electric eel, E.C. 3.1.1.7) assay for evaluating quaternary pyridinium oxime regenerators has been developed. Low density polyethylene beads (4 mm) were functionalized to a terminal aldehyde such that AChE could be immobilized via a Schiff base linkage [Noda et. al., Can. J. Biochem. 57, 1200 (1979)]. AChE activity in immobilized enzyme containing columns could be continuously monitored spectrophotometrically in a closed loop fashion using acetylthiocholine and dithiobis(nitrobenzoic acid) (DTNB). The system allowed for independent inactivation and reactivation of AChE followed by determination of AChE activity. The immobilized enzyme exhibited good esterase activity (0.4 units/bead) and the kinetics for substrate hydrolysis were flow-rate dependent. The immobilized enzyme retained its activity upon storage at -16°C for at least one month. The immobilized enzyme could be inactivated with diisopropylfluorophosphate (DFP) and reactivated to 50-70% of original activity with N-methylpyridinium-2-carbaldoxime iodide (2-PAM) (10^{-4} M) in less than 20 minutes. This system has advantages over previously published procedures because regenerator-catalyzed hydrolysis of substrate is minimized and deactivator-reactivator interactions are eliminated, subsequently, minimizing the need to correct experimental results. (This research was supported by DOD #DAMD17-82-C-2078).

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MECHANISM OF 5-HYDROXYMETHYLFURFURYLTRIMETHYLAMMONIUM TO INHIBIT THE RELEASE OF ACETYLCHOLINE FROM THE MOUSE CEREBRUM. Neelam Jaiswal* and B. V. Rama Sastry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

5-Hydroxymethylfurfuryltrimethylammonium (5-HMFT, 1.9 nM) inhibited the release of acetylcholine (ACh) from mouse cerebral slices. This inhibition was due to activation of muscarinic receptors (M2) which were blocked by scopolamine (10 nM) but not atropine (1 μM). It did not activate the muscarinic receptors (M1) in the smooth muscle. In view of the selectivity of 5-HMFT at M2 receptors, its mechanism for ACh release was studied. Mouse cerebral slices were incubated in a Krebs Ringer buffer containing (methyl ^{3}H)choline (0.1 mM; 0.25 μCi/ml) for 60 min. They were filtered, washed and transferred to a microbath set up for superfusion with the above buffer containing hemicholinium-3 (10 μM). The release of ^{3}H-ACh into the superfusate was measured. In a medium containing Ca^{++} (2.6 mM), 5-HMFT (1.9 nM) decreased both spontaneous and electrically evoked release of ACh by 50%. Its effect was not significant in Ca^{++} free medium. It decreased K^+ (20 mM) induced ACh release which was blocked by 5-HMFT. At 2.6 mM Ca^{++}, disteroylphosphatidic acid (DSPA, 43 μM) increased ACh release (40%) which was blocked by 5-HMFT. This effect was not reversed by increasing the Ca^{++} level to 5.96 mM. These studies indicate that 5-HMFT activates an M2 receptor and decreases Ca^{++} influx which is necessary for release of ACh. (Supported by US PHS-NIH grants AG-02077 and HD-10607 and The Council for Tobacco Research, U.S.A., Inc.)

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AF64A REDUCES ACETYLCHOLINESTERASE (AChE) STAINING, AND UNCOVERS AChE-POSITIVE CELL BODIES IN RAT HIPPOCAMPUS, IN VIVO. D.S. Arst*, T.W. Berger*, A. Fisher* and I. Hanin. Depts. Psychiatry and Biology, Univ. Pgh., Pittsburgh, PA 15213, and Israel Institute for Biological Research, Ness-Ziona, Israel

Recent reports have demonstrated the specific in vivo cholinotoxicity of AF64A (Fisher et al, 1982). We sought to extend these observations by a histochemical approach. Eight adult Sprague-Dawley rats were stereotaxically injected with AF64A (2nmol/2 ul), unilaterally, in the dorsal hippocampus. After 5 days, the animals were treated with DFP (1.5 mg/kg, i.m.), sacrificed 20 hours later, and perfused with 10% neutral buffered formalin. Tissue was processed for AChE staining according to Butcher and Bilezikjian (1975). AChE patterns of the non-injected hippocampus were identical to those described for normal tissue (Mosko et al., 1973). On the other hand, in the contralateral, AF64A-treated hippocampus, we observed a marked decrease in AChE staining near the injection site. The decreased density of AChE staining produced by AF64A revealed AChE-containing cell bodies in the hilus of the dentate gyrus. These results show that: 1) AF64A may selectively destroy AChE-containing nerve terminals; 2) under our experimental conditions, the effect of AF64A appears to be restricted to terminal, and not to somal regions of cholinergic neurons, and 3) using AF64A, we may have uncovered cholinergic interneurons in the hippocampus, such as those identified by Talbot and Butcher (1976). Neurochemical experiments to corroborate these anatomical results are currently in progress. #MH34893

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CHOLINESTERASE ACTIVITY IN THE INTACT SUPERIOR CERVICAL GANGLION OF THE RAT IS RESISTANT TO INHIBITION BY SOMAN. G.B. Viana*, S.S. Deshpande and F.C. Kauffman, University of Maryland School of Medicine, Baltimore, MD 21201.

Inhibition of cholinesterase (ChE) and recovery of this activity in various tissues of the rat were examined after a single s.c. injection of 60 μg soman/kg. Within 2 hours of treatment, activity was reduced by 60% in soleus muscles but not altered in extensor digitorum longus (EDL) muscles. At 24 hours after administration of the drug, ChE was decreased approximately 70% in soleus muscles and only 40% in EDL muscles. At this time, plasma ChE was depressed 50% and brain ChE by 80%. In contrast, activity of ChE in the superior cervical ganglion (SCG) was not significantly altered at any time period. Use of Iso-OMPA or BW284c51 indicated that both pseudocholinesterase and acetylcholinesterase in the intact ganglion in vivo were not inhibited by soman. Action of soman on the SCG in vivo differed from broken cell preparations of the SCG where soman caused a profound inhibition (half-inhibition, occurred with 0.5×10^{-9} M soman). Thus, ChE in broken cell preparations of the SCG but not the intact tissue is sensitive to the action of soman. Mechanisms responsible for this resistance are not known but may involve either the presence of hydrolytic enzymes or diffusion barriers preventing entry of the organophosphate into the tissue. Alternatively, the turnover of ChE may be very rapid in autonomic neural tissue. (Supported in part by a grant from USAMRDC.)

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RAT ADRENAL CATECHOLAMINES AND THEIR BIOSYNTHESIZING ENZYMES DURING OPIATE WITHDRAWAL. Peter S. DiStefano* and Oliver M. Brown* (SPON: I.M. Weiner). SUNY, Upstate Med. Ctr., Syracuse, NY 13210

Rat adrenals were assayed for catecholamine (CA) levels, tyrosine hydroxylase (TH), dopamine-β-hydroxylase (DβH) and phenylethanolamine-N-methyltransferase (PNMT) activities at 0, 6 hrs, 1, 3, 5 and 7 days after morphine withdrawal. Rats were made dependent on morphine with 75 mg morphine pellets implanted subcutaneously. Withdrawal was precipitated with naloxone (3 mg/kg, s.c.). CA, as well as TH and PNMT reaction products were assayed using HPLC-EC. DβH products were assayed spectrophotometrically. At 6 hrs post withdrawal adrenal EPI levels were 55% of control. EPI values recovered to normal by 3 days and showed a 15% increase over controls at 5 and 7 days. TH activity peaked at 1 day of withdrawal (250% of control) and declined to 120% of control at 7 days. DβH activities showed no elevations until day 3 of withdrawal. At day 5 adrenal DβH activity was 235% of control declining to 220% at day 7. No significant change in PNMT activity occurred at any time point of withdrawal at this dose. Clonidine (0.1-3.0 mg/kg, s.c.) was capable of inhibiting the decrease in adrenal EPI at 6 hrs of withdrawal. The ED_{50} for this response was 0.1 mg/kg and maximal inhibition of EPI release occurred at 1.0 mg/kg. Clonidine also attenuated the rise in adrenal TH activity at 6 hrs with the maximal response occurring at 0.1 mg/kg.

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